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13. ABSTRACT (Maximum 200 Words)

Mammary epithelial cells undergo periodic cycles of growth, differentiation and apoptosis during pregnancy and lactation. These processes are initiated by a complex series of signals that include mammotrophic hormones and locally-derived growth factors. This study determined the mechanism by which Ras activation, an important mitogenic signal transduction pathway that is frequently activated in breast carcinoma, inhibits mammary differentiation and apoptosis. We have demonstrated that the Ras pathway is activated by EGF stimulation of HC11 mammary epithelial cells. EGF stimulation results in activation of Erk and Akt signal transduction pathways and prevents lactogenic differentiation. Inhibition of either Ras, Erk or Akt can counter the effects of EGF on lactogenic differentiation. Expression of DNRas in HC11 cells enhances Stat5 phophorylation and DNA binding; this results in increased lactogenic differentiation as measured by elevated beta casein transcription, lipid synthesis and mammosphere formation. Using DNA microarray analysis global changes in gene expression were measured in HC11 cells undergoing lactogenic differentiation. Using the same technology genes whose expression was altered by EGF stimulation during differentiation were identified. This information provides an expression profile of gene regulation during lactogenic differentiation of HC11 cells, and identified novel targets in breast tissue exposed to mitogens.

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INTRODUCTION

Epidemiological studies indicate that the age at first pregnancy and lactation have an impact on later development of breast cancer. Mammary epithelial cells undergo periodic cycles of growth, differentiation and apoptosis during pregnancy and lactation. These processes are initiated by a complex series of signals which include mammotrophic hormones and locally-derived growth factors [1]. This study is aimed at determining the mechanism by which an important mitogenic signal transduction pathway, which is frequently activated in breast carcinoma [2,3], inhibits mammary differentiation and apoptosis.

There are limited human models of mammary differentiation available for study at the present time. However, the HC11 mouse mammary epithelial cells differentiate and synthesize β-casein following growth to confluency and stimulation with the lactogenic hormone mix, DIP (dexamethasone, insulin, prolactin) [4,5]. Regulation of β-casein expression in HC11 reflects *in vivo* regulation of this protein in the mammary gland [4]. Prolactin stimulation results in Jak2-mediated tyrosine phosphorylation of Stat5 a and b and nuclear translocation of the factors [6]. In HC11 cells the activation of Stat5 is not dependent on the Ras-Erk pathway [6] and, in fact, the induction of β-casein expression can be blocked by receptor tyrosine kinase signaling at the time of prolactin addition [7-10]. It is not clear which signal transduction pathways are responsible for the inhibition of β-casein synthesis by receptor tyrosine kinase signaling. However, the inhibition of β-casein expression by treatment of HC11 cells with EGF or Cripto [CR-1], an EGF family member, occurs through a Ras- and phosphotidylinositol-3-kinase (PI-3 kinase)-dependent mechanism [11]. Determination of the signaling mechanism(s) that are responsible for inhibiting differentiation will provide critical insight into control of this process in HC11 cells. Because inhibition of differentiation in HC11 cells appears to be dependent upon Ras, and possibly its association with PI-3-kinase, these studies focus attention on the role of Ras and its effectors in the differentiation of mammary epithelial tissue. We propose that the growth factor regulated inhibition of DIP-induced differentiation of HC11 cells results from the activation of Ras effector pathways in addition to Raf-Mek-Erk. Inhibition may require activation of the Ras-PI-3-kinase pathway and/or the Ras-RasGAP-Rho pathway.

We tested our hypothesis by constructing HC11 cell lines carrying dominant-negative (DN) Ras and HC11 cell lines expressing elevated levels of active Ras. These cell lines were used to dissect the control of differentiation using a series of markers for differentiation and cell cycle changes.

In addition, cDNA microarray analysis techniques was used to detect global changes in gene expression induced by differentiation in the HC11 cell background. We identified genes whose expression is specifically increased and decreased in these cells following induction of lactogenic differentiation. Genes whose expression was altered by exposure to EGF during lactogenic differentiation were also identified. This information provides an expression-based profile of gene regulation during lactogenic differentiation of HC11 cells, and it will be useful in identifying novel targets in breast tissue exposed to mitogens.

A complete understanding of the regulation of the differentiation process in mammary epithelial cells will aid in understanding the cellular changes and mechanisms leading to carcinogenesis in this tissue and allow evaluation of therapeutic strategies on the differentiation process.

BODY

The majority of the work completed during this period addressed the goals in the original statement of work as opposed to the revised statement of work for this project. Hence, the results reported here primarily address the original statement of work.

Task 1. Construction of vectors and cell lines. This is described in detail in the manuscript enclosed as Appendix item #1.

Construction of HC11 Tet-Off cell lines. The HC11 cell line was transfected with the pTetOff plasmid (Clontech) and the transfected cells were selected for 10 days with G418 (200-500μg/ml). Then individual colonies were picked, expanded and screened for ability to regulate a Tet-promoter. This was accomplished by transfection with a Tet-promoter luciferase construct and assay for luciferase activity with and without Doxicyclin (0-0.5-2.0μg/ml). Several of the transfected cell lines, Ax-TetOff and C6-TetOff, contained a TRE that could be regulated by Dox. These cell lines (HC11-Tet Off) were used to construct lines for the regulated expression of activated Ras or dominant negative Ras.

Production of Retroviral vector Stocks and infection of HC11 cells. pREV-TRE, a retroviral vector that expresses a gene of interest from Tet-responsive element (TRE), was derived from pLNCX, a Moloney murine leukemia virus (MoMuLV)-derived retroviral vector. The 5' viral LTR controls expression of the transcript that contains φ^+ (the extended viral packaging signal), and the hygromycin resistance (Hyg^r) gene for antibiotic selection in mammalian cells. pRevTRE also includes the *E. Coli* Amp^r gene for antibiotic selection in bacteria. The internal TRE contains seven direct repeats of the 42-bp tetO operator sequence, upstream of a minimal CMV promoter. This promotor was used to inducibly express the genes of interest in response to varying concentrations of Doxicyclin (Dox). TtA binds to the Tet-response element (TRE) and activates transcription from the minimal promoter in the absence of Dox. The plasmids pREV-TRE-RasV12 (active K-Ras 2BV12) and pREV-TRE -RasN17 (Dominant Negative K-Ras 2B(N17) were constructed by introduction of K-Ras cDNA into pREV-TRE.
Retroviral vector stocks of pRev-Tre, pRevTre-RasV12, pRevTre-RasN17 were prepared and used for retroviral infection of HC11-TetOff cells. The HC11-TetOff cell line was infected and selected in hygromycin and Doxicyclin (2μg/ml) for ten days. Six colonies were picked from Tet-Off pREV-TRE, pREV-TRE-RasV12 and pREV-TRE-RasN17 plates and seeded in 24 well plates. These cells was expanded and tested with or without Dox for the presence or absence of Ras RNA by Northern Blot.

Task 2 and 3. Determination of the effect of dominant negative Ras expression on differentiation and Stat5 activation.

EGF blocks hormone-induced HC11 differentiation through Mek and PI-3-kinase-dependent pathways. Previous studies have demonstrated that EGF blocked lactogenic hormone-induced differentiation of HC11 cells [7], and recent data suggests that this block required Ras and PI-3-kinase activity [11]. In the present study specific chemical inhibitors of signal transduction pathways were used to further analyze the contribution of individual signaling pathways to the block of HC11 differentiation by EGF. Because lactogenic hormone-induced differentiation of

HC11 cells is characterized by the initiation of β-casein transcription, the HC11-luci cell line, which contains a β-casein promotor linked to the luciferase gene, was used to provide a rapid readout of the differentiation process.

The HC11-luci cells were induced to differentiate with DIP in the absence and presence of EGF. Specific inhibitors of Mek, and PI-3-kinase were added to cells at the time of induction of differentiation. As expected there was a significant inhibition of β-casein driven luciferase activity in the EGF-treated samples compared to the DIP control. However, several compounds (PD98059, LY294002 and wortmannin) restored the β-casein promotor driven luciferase activity that was blocked by EGF (Figure 1A). The results demonstrated that the inhibition of Mek-Erk signaling by PD98059 and PI-3-kinase signaling by LY294002 and wortmannin disrupted the EGF signaling that inhibited lactogenic hormone-induced differentiation, as measured by the activation of β-casein promotor driven luciferase expression.

The effect of chemical inhibitors of signal transduction pathways on the synthesis of β-casein RNA was examine (Figure 1B). The results confirmed that exposure of HC11 cells to DIP activated β-casein expression and that EGF blocked the expression. However, inclusion of PI-3-kinase or Mek1 inhibitors in the induction media with EGF reversed the EGF-induced inhibition of endogenous on the b-casein promotor activity in the HC11-luci cells.

In addition, the treatment of HC11 cells with DIP resulted in increased Stat5 DNA binding, and previous studies demonstrated that the DNA binding activity of Stat5 was reduced by the simultaneous addition of EGF and lactogenic hormones to HC11 cells [Marte, 1995 #510]. EMSA was performed to examine the ability of the signal transduction inhibitors to alter Stat5 DNA binding. Nuclear extracts were prepared from HC11 cells induced to differentiate in the presence of Jak2, Mek1 or PI-3-kinase inhibitors. The results indicated that prolactin stimulation in the presence of the Mek1 and PI-3-kinase inhibitors enhanced Stat5 binding to DNA compared to the binding detected with prolactin alone (Figure 2A). In contrast, exposure of the HC11 cells to prolactin plus AG490, an inhibitor of Jak2 tyrosine phosphorylation, inhibited Stat5 DNA binding (Figure 2A, lanes 4 and 8). The results in figure 1 indicated that Mek1 and PI-3-kinase inhibitors restored the prolactin-induced Stat5 promotor activity inhibited by EGF. Moreover, the same Mek and PI-3-kinase inhibitors enhanced Stat5 DNA binding. Blocking the Mek-Erk and PI-3-kinase pathways with specific inhibitors both enhanced HC11 differentiation and prevented the EGF-dependent disruption of HC11 differentiation.

HC11 cells expressing dominant negative (N17) Ras exhibit an enhanced differentiation response. Ras activation likely regulates the activation of the Erk pathway by EGF and possibly contributes to the activation of PI-3-kinase. Hence, the role of Ras activation in the disruption of HC11 differentiation by EGF was examined further. HC11 cell clones expressing either activated Ki-Ras (V12) or dominant negative (DN) Ki-Ras (N17) were constructed as described in Materials and Methods. The HC11 cell lines constructed contained the Ras cDNAs under the control of a Tet-responsive promotor in a Tet-off system. Hence, the expression of Ras increased following the removal of doxycycline from the culture media. Several independent clones containing each vector were isolated and characterized for the inducibility of Ras gene expression following the removal of doxycycline from the cultures. As expected, the inducibility varied for the individual Ras(V12) and DN Ras clones. The results obtained with three independent clones derived from each vector are shown in Figure 3.

The DN Ras and the Ki-Ras(V12) HC11 cell lines were compared to the vector control cell line, REV-TRE, to determine the effect of the Ras gene expression on lactogenic hormone-induced differentiation. HC11 transfectant cell lines expressing dominant negative Ras(N17) or activated Ras(V12) along with the vector control cell line were grown for 72 hours in the absence of doxycycline at which point the confluent cultures were incubated in DIP differentiation media. RNA was harvested from cells at 0, 48, and 72 hours post addition of DIP and used to determine the level of Ras and β casein expression by Northern blotting. The results in Figure 3 indicated that Ki-Ras(V12) expression inhibited β-casein expression by approximately 50% compared to the TRE control cell line. In contrast, the expression of dominant negative Ras(N17) enhanced β-casein induction up to two-fold compared to the control. The results demonstrated that the amount of N17 Ras expression correlated with the effect on differentiation. The HC11 cell clone expressing the greatest amount of Ras N17 (clone 12) exhibited the greatest level of β-casein expression.

In parallel experiments the effect of Ras expression on the prolactin-induced tyrosine phosphorylation of Jak2 and Stat5 was examined. HC11 TRE vector control cells as well as the Ki-Ras(V12) clone 1 and DN Ras(N17) clone 12 cells were stimulated with prolactin and the phosphorylation status of the Stat5 protein was determined by immunoprecipitation and Western blotting using anti Stat5 tyrosine 694 (Y694) phosphorylation site-specific antibodies. The results, seen in Figure 4B, indicated that the tyrosine phosphorylation of Stat5 was enhanced and sustained in the DN Ras(N17) HC11 cell line compared to the TRE vector control cell line. However, the tyrosine 694 phosphorylation was of a shorter duration in the cell lines expressing activated Ki-Ras(V12) than in the TRE control cells. These results suggested that Ras-dependent signal transduction can modulate Stat5 phosphorylation in HC11 cells in response to prolactin. The Stat5 EMSA results supported this conclusion (Figure 4C). Enhanced Stat5 DNA binding in response to prolactin stimulation was observed in the DN Ras(N17) HC11 cell lysates as compared to the vector control. In contrast, the Stat5 DNA binding activity was reduced in cells expressing activated Ki-Ras(V12). In conclusion, an increase in HC11 cell lactogenic hormone-induced differentiation is observed following blockade of the Ras signaling pathway. Moreover, in the HC11 cells that have Ras activity blocked, the enhancement of hormone-induced differentiation appeared to be attributable to an increase in Stat5 tyrosine phosphorylation and to an increase in Stat5 DNA binding resulting in enhanced transcription of β-casein, a Stat5-regulated gene.

Infection of HC11 cells with DN Ras adenovirus enhances lactogenic differentiation. Infection of cells with replication defective adenovirus encoding dominant negative Ha-Ras(N17) was used as another mechanism to examine the influence of the Ras pathway on lactogenic differentiation. HC11 cells and HC11-luci cells were infected with 10 MOI of either replication defective control adenovirus or adenovirus encoding DN (N17) Ras. At 24 or 48 hours post infection the cells were examined for the effect of DN Ras on Stat5 phosphorylation, β casein promotor activity and β casein RNA levels. As demonstrated in Figure 5A HC11-luci cells infected with control virus or DN Ras virus were stimulated with DIP and the level of Stat5 tyrosine 694 phosphorylation was determined. The results indicated that the expression of DN Ras (N17) increased the level of Stat 5 phosphorylation in response to DIP compared to either uninfected or vector control-infected cells. HC11-luci cells infected with either replication defective control adenovirus or adenovirus encoding DN Ras (N17) were tested for activation of β-casein promotor-driven luciferase activity (Figure 5B). There was a five-fold increase in the activation of luciferase activity in the DN Ras (N17) cells compared to the uninfected cells or the control adenovirus infected cells. In addition, there was some activation of luciferase activity in cells infected with the DN Ras (N17) virus without DIP exposure. This result was reproducible and is not seen when uninfected cells or vector infected cells were exposed to DIP. Finally, RNA from HC11 cells infected with either replication defective control adenovirus or adenovirus encoding DN Ras (N17) was tested for expression of β-casein following exposure to DIP for 24 or 48 hours. The results in Figure 5C indicated that the infection with DN Ras (N17) virus resulted in a two-fold increase in β-casein RNA compared to the uninfected or vector infected cells exposed to DIP.

HC11 cells expressing dominant negative (N17) Ras exhibit reduced response to EGF. Studies were performed to determine if the DN Ras (N17) expression could block EGF-induced responses in stable transfectants of HC11 cells. HC11 cells respond mitogenically to EGF. The TRE vector control cells and the DN Ras (N17) cells were stimulated with EGF and the ability of the cells to proliferate was examined using the MTT assay. The results demonstrated that the DN Ras (N17) cell line was growth inhibited by 40% in both the absence and presence of EGF compared to the vector control cell line. This experiment was repeated using TGFα treatment of HC11 vector control and DN Ras (N17) cells. Again, the DN Ras (N17) cells exhibited a lower response to EGF and TGFα than did the vector control cell line. (Figure 6)

The ability of DN Ras to prevent the disruption of lactogenic hormone-induced differentiation by EGF in HC11 cells was examined. The cells were exposed to lactogenic hormone differentiation media in the presence and absence of EGF for varying lengths of time, RNA was extracted and the level of β-casein mRNA was analyzed by Northern blotting. The results in Figure 6 demonstrated that EGF did not inhibit the induction of β-casein transcription in response to DIP treatment in the DN Ras (N17) cell line and, hence, it appeared that differentiation proceeded in these cells even in the presence of EGF. In contrast, the vector control cell line did not express β-casein RNA in the presence of DIP plus EGF. These results demonstrated that DN Ras expression prevented the disruption of hormone-induced differentiation by EGF in HC11 cells.

HC11 cells expressing dominant negative (N17) Ras exhibit reduced Erk activation in response to EGF.

HC11 cells expressing DN Ras(N17) were examined to determine if expression of DN Ras prevented the activation of Mek-Erk or PI-3-kinase signaling in response to EGF. In Figure 7 the stable transfectants were removed from doxycycline and grown to confluence. The cells were starved and then stimulated with EGF for varying amounts of time. Cell lysates were prepared and analyzed by Western blot using antibodies that detect phosphorylated forms of different signaling proteins. The results revealed that stimulation of HC11 vector control cells with EGF resulted in activation of p44Erk as detected by reactivity with an antibody that recognizes the active phosphorylated form of Erk. In contrast, in HC11 cells expressing DN Ras (N17) there was no activation of p44Erk, although the Erk protein levels in the cells were similar to those in the vector control cells. The analysis of other signaling proteins revealed that Akt was activated in the control HC11 cells and partially attenuated in the DN Ras HC11 cells following treatment with EGF. This demonstrated that the PI-3-kinase pathway was not completely blocked by DN Ras expression in HC11 cells. Moreover, activation of Jun kinase and p38 kinase by EGF was not deficient in the N17 Ras HC11 cells (data not shown). These results suggest that the Mek-Erk pathway was most sensitive to inhibition by DN Ras expression.

Cells infected with the control adenovirus vector or adenovirus encoding DN Ras (N17) were examined for the effect of EGF on signal transduction pathways in an analogous fashion. The results in Figure 7 demonstated that DN Ras (N17) adenovirus also blocked the activation of Erk but not the phosphorylation of AKT on serine 473, used as a measure of PI-3-kinase activity. The results from the DN Ras(N17) expressing cells indicates that blocking the Ras pathway in this manner in HC11 cells primarily blocks signaling to the Raf-Mek-Erk pathway. Hence, these data support the conclusion that in HC11 cells activated Ras(V12) inhibits β-casein transcription via Mek-Erk signaling, and that the effect of DN Ras(N17) expression on β-casein is primarily a result of its inhibition of the Mek-Erk pathway.

Task 5, 6, 9. DNA Microarray analysis of changes in gene expression following induction of lactogenic differentiation.

Cell preparation. HC11 mouse mammary epithelial cells were cultured in RPMI 1640 medium containing 10% fetal calf serum, 5 μg/ml Insulin, 10 mM Hepes and 10 ng/ml epidermal growth factor(EGF). Cells were maintained in T75 flasks after confluence for 4 days, then starve the cells in the media without EGF for 24 hours. The cells were then incubated in differentiation media(serum containing RPMI with dexamethazone(10^{-6} M), insulin(5 μg/ml) and prolactin(5 μg/ml) for 72 hours, undifferentiated HC11 cells were used as control. The cells were scraped from the flasks and precipitated for microarray RNA extraction.

RNA preparation. RNAs were extracted using Trizol reagent (Invitrogen) and RNeasy maxi kit (Qiagen). Wash the cells in the flask once with PBS. Add 5 ml of Trizol to a 75 cm^2 flask (about 2×10^7 cells) and mix by rotating. Add 2/10 volume of chloroform and shake for 15 seconds. Centrifuge at 12,000g for 15 minutes at 4°C. Take off the supernatant and add it to a polypropylene tube, recording the volume of the supernatant. Then 0.53 volumes of ethanol were added to the supernatant slowly while vortex, this step produced a final ethanol concentration of 35%. Add the supernatant from an extraction to an RNeasy maxi column, which is seated in a 50 ml centrifuge tube. Centrifuge at 2880g in a clinical centrifuge with a horizontal rotor at room temperature for 5 minutes. Pour the flow-through back onto the top of the column and centrifuge again. Discard the flow-through and add 15 ml of RW1 buffer to the column, centrifuge at 2880g for 10 minutes. Discard flow-through then add 10 ml of RPE buffer and centrifuge at 2880 g for 10 minutes. Discard flow-through and add another 10 ml of RPE buffer and centrifuge at 2880g for 15 minutes. Put the column in a fresh 50 ml tube and add 1 ml of DEPC treated water from the kit to the column and lets stand for 1 minute, centrifuge at 2880g for 5 minutes. Repeat this process once. Concentrate samples to greater than 1 mg/ml by centrifugation on a MicroCon 100 filter unit at 500g. Determine the concentration and ratio of RNA in the concentrated sample by spectrophotometry. Store at -80°C. Or purify RNA to get mRNA using Oligtex mRNA kit.

Labeling, hybridization and analysis. Gene expression analysis was performed using mouse NIA(15K) oligonucleotide slides for microarray experiments (Agilent). In addition, Atlas Glass Mouse 3.8 Microarrays(Clontech Laboratories), which include 3800 mouse DNA oligo probes, a list of these genes is available at the Clontech web site (http://www.clontech.com/atlas/genelist/index.shtml). Fluorescent labeling of RNAs was performed by using an Atlas Glass fluorescent labeling kit (Clontech Laboratories) according to manufacturer's manuals. Synthesized first-strand cDNAs from RNA of HC11 cells with and without differentiation were labeled with fluorescent dyes, Cy3 and Cy5 (Amersham Pharmacia Biotech), respectively. The labeling was switched during experiment, i.e. differentiation group was labeled with Cy3 two times, and Cy5 two times; and the control group was labeled with Cy5 two times, and Cy3 two times, vise versa. The quality of the labeling and the amount of each probe used were determined by absorbance measurement for Cy3 and Cy5 probes in a Beckman DU-600 scanner. Hybridization of the microarrays was carried out in a hybridization solution for 16 hours at 50°C. Then wash the slide with wash solution for 3 times provided by manufacturer. The microarray slides were scanned and analyzed by using a GenePix 4000B scanner in both Cy3 and Cy5 channels. The differentiation induced gene up- or down-regulations were obtained by dividing differentiation value over control value. The average of Cy3 and Cy5 signals from nine house-keeping genes gives a ratio which was used to normalize the individual signals. The data is included as Figure 8.

Statistical analysis. Normalization and analysis of the gene expression profiles was performed as follows:
Exclude the spot if red and green intensity is below 30. Normalize (center) each array using median over entire array. Truncate intensity ratios (and inverse ratios) greater than 64. Exclude a gene under any of the following conditions: Less than 20 % of expression data have at least a 1.5 -fold change in either direction from gene's median value. Percent of data missing or filtered out exceeds 50 %.
In DIP vs control experiments, there are 10813 genes that passed filtering criteria in total of 20280 genes. And the first 2479 genes are significant at the nominal 0.05 level of the paired T-test.
In EGF plus DIP vs DIP experiments, there are 1386 genes passed filtering criteria in total of 20280 genes. And the first 1129 genes are significant at the nominal 0.05 level of the paired T-test.

Generation of probes. Using accession number of interested gene to find out the mRNA sequence at internet, design primers for RT-PCR about 200-500 bp gene which can be used as a probe. Use Gene Amp RT-PCR kit (Roche) to amplify the cDNA and insert the correct-sized fragment into a pCR2.1 TA cloning kit(Invitrogen), candidate clone was sent to sequencing to prove the correct sequence. Double strand DNA of the insert was digested from pCR 2.1 plasmid, gel purified as a probe.The probes were used for hybridization to Northern blots containing RNA from HC11 cells undergoing lactogenic differentiation.

Verification of gene expression by Northern blot. HC11 cells were treated in the same way as for microarray experiment, and then induced to differentiated for 12 h, 24 h, 48 h, 72 h, 96 h, 120 h, and 144 h, respectively; undifferentiated cells at 0 h, and 144 h were used as controls. In other experiments RNA from cells induced to differentiate for 72 hours in the presence or absence of EGF (10μg/ml) were compared to RNA from undifferentiated control cells. RNA samples (10 μg) were electrophoresed on agarose gels and transferred to nylon filters. The filters were reacted with labeled probes in hybridization solution and incubated overnight. The blots were washed and expose to X-ray film and then quantitated on a beta scanner. Beta-actin probe was used to hybridize the same membrane and then scanned to get a normalized data. Genes that exhibit increases in expression during differentiation are shown in figure 10.

Oligonucleotide microarrays containing approximately 20,000 genes (Agilent) and 3,800 genes (Clontech) were used in this study. The gene expression patterns of the regulated genes were verified by Northern blot analysis. Data were analyzed by using a NIH program (BRB Array Tools). Microarray expression analysis and Northern blot data indicate that lactogenic differentiation induced multiple gene transcription in HC11 cells, while the presence of EGF blocked most of these transcription processes. Normalization and analysis of the gene expression profiles was perfomed. In DIP vs control experiments, there are 10813 genes that passed filtering criteria from a total of 20280 genes. The first 2479 genes are significant at the nominal 0.05 level of the paired T-test. In EGF plus DIP vs DIP experiments, there are 1386 genes passed filtering criteria in total of 20280 genes. The first 1129 genes are significant at the nominal 0.05 level of the paired T-test.

By using microarrays from different sources and performing additional northern blots the reliability of the array data has been demonstrated. Statistical analysis and the use of additional analysis tools have allowed us to draw conclusions from the data regarding the role of EGF in blocking lactogenic differentiation. Some of the conclusions include the following. Numerous ribosomal protein transcripts were induced by stimulation with lactogenic hormone even in the presence of EGF, suggesting that these genes are not a target of the mitogen-induced inhibition. Differentiation also induced gene expression changes in cell cycle regulators. Data showed that while cyclin D1 transcription was inhibited as predicted, cyclin G2 and cyclin-dependent kinase inhibitor were induced during differentiation. $P21^{CIP}$ was known to be induced following initiation of lactogenic differentiation, but no data was available concerning cyclin G2. In addition, the transcription element binding proteins KLF4, KLF6, KLF7 and KLF9(BTEB1) were induced during lactogenic differentiation but were inhibited by EGF. This provided, in part, an explanation of the large scale gene expression changes during lactogenic differentiation that were blocked by EGF. Interestingly, KLF6 was recently proposed to be a tumor suppressor gene. Therefore, the increased transcription and possible expression of this gene might explain the decreased morbidity of breast cancer in women who had early pregnancy and lactation. Our current studies are directed toward understanding the contribution of some of the genes whose expression is regulated during lactogenic differentiation. We are expressing cDNAs encoding a number of genes in HC11 cells to test the effect of expression on lactogenic differentiation. These include KLF 6 and 9, cyclin G2, connective tissue growth factor, serum glucocortecoid kinase.

Analysis of changes in gene expression that occur in cell lines expressing dominant negative Ras and dominant negative Mek1 as well as the activated versions of those genes have been initiated. The results are being compared to those obtained for HC11 control cell line following exposure to lactogens.

Task 10,11

Primary cultures and explant cultures from mouse mammary glands have been established for additional experiments. These cultures have been exposed to growth factor and RNA has been isolated for expression profiling. Adenoviral vectors expressing dominant negative versions of Ras and Mek1 have been isolated and purified replication defective adenovirus has been prepared. This is being used to infect primary cell cultures of mouse mammary glands in order to determine if the kinase pathways inhibited in HC11 cell culture are similarly affected in primary cells. In addition, RNA has been isolated from infected cultures for microarray studies.

KEY RESEARCH ACCOMPLISHMENTS

-Construction of HC11 mouse mammary epithelial cells capable of regulating a tetracycline-inducible promotor.

-Construction of HC11 cell lines expressing RasV12 and RasN17 under the control of a regulatable promotor.

-Demonstartion that EGF disrupts differentiation via stimulation of the Erk and Akt pathways.

-Demonstration that DNRas adenovirus can be used to infect HC11 cells and that DNRas expression enhances activation of the β casein promotor.

-Detection of a set of genes that is expressed at 2-fold or greater levels during lactogenic differentiation of HC11 cells.

- Detection of a set of genes whose expression decreased 2-fold during lactogenic differentiation of HC11 cells.

-Detection of genes whose expression increased or decreased as a result of exposure to EGF during differentiation.

REPORTABLE OUTCOMES

- Construction of HC11 mouse mammary epithelial cells capable of regulating a tetracycline-inducible promotor.
- **publication**: Cerritto MG, Galbaugh T, Chopp T, Wang W, Salomon D and Cutler ML. Dominant negative Ras enhances lactogenic hormone-induced differentiation by blocking activation of the Raf-Mek-Erk signal transduction pathway. Journal of Cellular Physiology, (epublished March 29, 2004) D0I: 10.1002/jcp.20077.

CONCLUSIONS

We have demonstrated that the Ras pathway is activated by EGF stimulation of HC11 mammary epithelial cells. This occurs in part via the increase in GTP-bound Ras in the cells. EGF stimulation results in activation of Erk, Akt and other signal transduction pathways and prevents lactogenic differentiation. Inhibition of either Ras (via DNRas expression) or Erk (via PD98059) or Akt (via wortmannin) can counter the effects of EGF on differentiation. The mechanism of disruption of differentiation appears to involve interference with the growth arrest that occurs prior to the induction of differentiation; the mechanism for growth arrest may require the downregulation of Mek1 expression. In addition, EGF mitogenic stimulation also inhibits Stat5 binding to its DNA binding site in the β casein promotor.

This data focuses on the role of two Ras effector signal transduction pathways (Erk and Akt) in preventing mammary epithelial cell differentiation. Our results indicate that inhibition of either or both of the pathways blocked the disruption of differentiation by mitogens of the EGF family. However, the block in signal transduction that resulted from dominant negative Ras expression inhibited the Mek-Erk signal transduction pathway and this inhibition is responsible for the effect on lactogenic differentiation. This approach to regulating differentiation may be useful in designing therapeutic approaches using signal transduction inhibitors (STIs).

A list of genes transcriptionally regulated during lactogenic differentiation has been identified. By using microarrays from different sources and performing additional northern blots the reliability of the array data has been demonstrated. Statistical analysis and the use of additional analysis tools have allowed us to draw conclusions from the data regarding the role of EGF in blocking lactogenic differentiation. Using this list and additional data from future expression profiling experiments, novel pathways important to the regulation of lactogenic differentiation will be identified.

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APPENDIX

The figures cited in the body of the report and the figure legends are contained in the attached Appendix.

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Dominant Negative Ras Enhances Lactogenic Hormone-Induced Differentiation by Blocking Activation of the Raf–Mek–Erk Signal Transduction Pathway

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Epidermal growth factor (EGF) and Ras mitogenic signal transduction pathways are frequently activated in breast carcinoma and inhibit mammary differentiation and apoptosis. HC11 mouse mammary epithelial cells, which differentiate and synthesize β-casein following growth to confluency and stimulation with lactogenic hormones, were used to study EGF-dependent signaling during differentiation. Blocking Mek–Erk or phosphotidylinositol-3-kinase (PI-3 kinase) signaling with specific chemical inhibitors enhanced β-casein promotor-driven luciferase activity. Because EGF stimulation of HC11 cells resulted in the activation of Ras, the effect of activated Ras (RasV12) or dominant negative (DNRasN17) on lactogen induced differentiation was examined. HC11 cell lines expressing RasV12 or DNRasN17 under the control of a tetracycline (tet)-responsive promotor were constructed. Activated RasV12 expression resulted in reduced tyrosine phosphorylation of Stat5 and a delay in β-casein expression in response to prolactin. However, the expression of tet-regulated DNRasN17 and adenovirus-encoded DNRasN17 enhanced Stat5 tyrosine phosphorylation, Stat5 DNA binding, and β-casein transcription. The expression of DNRasN17 blocked the activation of the Mek–Erk pathway by EGF but did not prevent the phosphorylation of AKT, a measure of activation of the PI-3-kinase pathway. Moreover, the expression of DNRasN17 prevented the block to lactogenic differentiation induced by EGF. Stimulation of HC11 cells with prolactin resulted in the association of the SHP2 phosphatase with Stat5, and this association was prevented by DNRasN17 expression. These results demonstrate that in HC11 cells DNRas inhibits the Mek–Erk pathway and enhances lactogenic hormone-induced differentiation. This occurs, in part, by inhibiting the association of the SHP2 phosphatase with Stat5. J. Cell. Physiol. 9999: 1–15, 2004. © 2004 Wiley-Liss, Inc.

Mammary epithelial cells undergo periodic cycles of growth, differentiation, and apoptosis during pregnancy and lactation. A complex series of signals that include mammotrophic hormones, locally derived growth factors and stroma initiate and regulate these processes. In this study, we address the problem of inhibition of mammary cell differentiation by mitogenic growth factors, including epidermal growth factor (EGF), that are found locally in the mammary gland. Because elevated levels of different growth factors in the EGF family such as transforming growth factor α (TGFα) and amphiregulin have been reported in breast tumors (Dotzlaw et al., 1990; Mizukami et al., 1991; Salomon et al., 1999) this study addresses an important issue in both normal development and neoplasia.

The HC11 mouse mammary epithelial cell line used in this study was derived from the COMMA-1D cell line, which was established from a midpregnant BALB/c

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mouse mammary gland (Danielson et al., 1984). This cell line has been employed as a model system for the study of regulation of mammary differentiation both in vitro and in vivo. HC11 cells introduced into the mammary fat pad differentiate into ductal-like structures (Humphreys and Rosen, 1997). In culture, HC11 mouse mammary epithelial cells differentiate and synthesize β-casein following growth to confluency and stimulation with the lactogenic hormone mixture, DIP (dexamethasone, insulin, prolactin) (Ball et al., 1988). β-casein expression in HC11 cells has been used as marker of differentiation, and its regulation in HC11 cells in culture reflects the in vivo regulation of expression of this protein in the mammary gland during pregnancy by prolactin (Ball et al., 1988; Peterson and Haldosen, 1998). Production of β-casein in cell culture is dependent upon both the presence of specific mitogens during the growth of the HC11 cells, cell–cell contact, deposition of extracellular matrix, and the subsequent prolactin-dependent activation of Stat5a and b when the cells have reached confluency (Taverna et al., 1991; Marte et al., 1995; Merlo et al., 1996). Prolactin stimulation results in Jak2-mediated tyrosine phosphorylation of Stat5a and b and nuclear translocation of these factors (Gouilleux et al., 1994; Marte et al., 1995; Han et al., 1997; Ali, 1998). In HC11 cells, the activation of Stat5 by prolactin is not dependent on the Ras–Erk pathway (Wartmann et al., 1996). However, the hormone-induced expression of β-casein can be blocked by the activation of different tyrosine kinase signaling pathways at the time of prolactin addition (Hynes et al., 1990; Marte et al., 1995; Merlo et al., 1996; Peterson and Haldosen, 1998). Previous studies have demonstrated that EGF prevents HC11 differentiation in response to lactogenic hormones. However, several signal transduction pathways have been implicated as responsible for the inhibition of β-casein synthesis. One study reported that the EGF-dependent inhibition of β-casein expression occurred through a Ras- and phosphoinositol-3′-kinase (PI-3 kinase)-dependent mechanism, not a Ras–Erk pathway (DeSantis et al., 1997; Salomon et al., 1999). More recently PTP-PEST, a phosphatase that can act on Jak2, was implicated as an EGF-induced protein contributing to this inhibition (Horsch et al., 2001).

Receptor tyrosine kinase (RTK) activation through different growth factor receptors leads to activation of Ras by guanine nucleotide exchange factors. The ErbB family of RTKs use this mechanism to stimulate signal transduction in the Ras pathway (Janes et al., 1994). Signal transduction that is downstream of Ras depends on the association of Ras GTPase with its effector proteins. Several proteins have been identified which associate with Ras in a GTP-dependent manner. These include Raf-1, RasGAP, p110 subunit of PI-3-kinase, AF6, Rin-1, Mek kinase 1, protein kinase C zeta, and RalGDS (Moodie et al., 1993; Van Aelst et al., 1993; Warne et al., 1993; Kikuchi et al., 1994; Rodriguez-Vicania et al., 1994; Akasaka et al., 1996). Activation of Ras initiates a signaling cascade via activation of the Raf-1 and Mek-1 kinases resulting ultimately in the activation of Erk kinases (Shibuya et al., 1992; Moodie et al., 1993). The results of several studies have indicated that the activation of the Ras–Erk kinase pathway can either induce or enhance the differentiation of breast cancer cell lines (Bacus et al., 1992; Giani et al., 1998; Lessor et al., 1998). However, the activation of the Ras–Raf–Mek–Erk pathway by EGF inhibits hormone-induced differentiation in HC11 cells (Hynes et al., 1990), and the expression of v-Raf, which also activates Erk signaling, has a similar effect (Jehn et al., 1992; Happ et al., 1993).

In the present study, we have addressed the mechanism of EGF inhibition of HC11 lactogenic hormone-induced differentiation by examining the involvement of specific signal transduction pathways on differentiation. These studies indicated that the Ras–Mek–Erk pathway and, to a lessor degree, the PI-3 kinase pathway contribute to this inhibition by EGF. Moreover, the expression of DN Ras prevented the EGF-dependent disruption of HC11 differentiation indicating that Ras-signaling is central to this process. DN Ras expression blocked EGF-induced activation of the Mek–Erk signaling but not PI-3-kinase signal transduction, indicating that stimulation of the Mek–Erk pathway is the primary mechanism blocking lactogenic differentiation in HC11 cells.

MATERIALS AND METHODS

Cell culture

Mouse mammary epithelial cell lines, HC11 and HC11-luci, kindly provided by Dr. Nancy Hynes, were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 5 μg/ml Insulin, 10 mM HEPES, and 10 ng/ml EGF as described (Hynes et al., 1990; Marte et al., 1995).

Lactogenic hormone-induced differentiation

The HC11 cells were grown to confluence and maintained for 3–5 days in RPMI 1640 medium supplemented with 10% fetal calf serum, 5 μg/ml Insulin, 10 mM HEPES, 10 ng/ml EGF to establish competence (Ball et al., 1988; Taverna et al., 1991). To induce lactogenic hormone-induced differentiation EGF-containing media was removed, the cells were rinsed twice and then incubated in differentiation media, i.e., serum free- or serum containing-RPMI with dexamethazone (10^{-6} M), insulin (5 μg/ml), and prolactin (5 μg/ml) referred to as DIP. The cells were harvested at the stated times after the addition of DIP. Alternatively, HC11 Tet-off cell lines were grown to confluence for 6 days in EGF-containing media in the absence of doxycycline, then maintained for 24 h in media without EGF prior to the addition of DIP. HC11 differentiation was characterized in these cells by the formation of domed structures referred to as mammospheres (Blatchford et al., 1995; Humphreys and Rosen, 1997) which were enumerated by phase contrast microscopy. The cell cultures were photographed using 20× objective with a Nikon Ix70 camera.

Construction of cell lines

The HC11 cell line was transfected with pTetOff plasmid (Clontech[Q1]) using Lipofectamine 2000 (Life Technologies[Q2]). The cells were incubated in G418 (200–500 μg/ml) selection media for 10 days, individual colonies were picked with cloning cylinders and expanded in 24-well plates. The colonies were screened for the regulation of the Tet-promoter by transfection with a Tet-promoter-luciferase construct and incubation in medium with and without doxycyclin (0–0.5–2.0 μg/ml). The promoter activity was assessed using a luciferase assay system (Promega[Q3]) with the light emission measured in a luminometer and expressed as light intensity/μg cell protein. Two cell lines exhibited up to 40-fold increase in a Tet-responsive promoter in response to the removal of doxycycline from the cultures. These HC11 tet-off cell lines were used for the construction of cell lines expressing specific genes under the control of the Tet-responsive element (TRE).

The HC11 Tet-off cell lines were infected with retroviral vectors expressing Tet-regulated *Ki-Ras* genes. pREV-TRE (Clontech), a retroviral vector that expresses a gene of interest from TRE, was derived from pLNCX, a Moloney murine leukemia virus (MoMuLV)-derived retroviral vector. The TRE contains seven direct repeats of the 42-bp tetO operator sequence, which can be bound by tTA transactivators, upstream of a minimal CMV promoter. The 5′ viral LTR regulates expression of the transcript that contains the viral packaging signal and the hygromycin resistance (Hyg^r) gene. The TRE is an internal promotor in this vector. pREV-TRE was used to inducibly express the Ki-Ras genes in response to removal of doxycyclin (Dox).

pREV-TRE-RasV12 (active K-Ras 2B-V12) and pREV-TRE-DNRasN17 (dominant negative K-Ras 2B-N17) plasmids were constructed by introduction of K-Ras cDNA into pREV-TRE plasmid and selection on hygromycin. For the production of retroviral vector stocks 1.5×10^5 PA317 packaging cells were transfected with 1 μg of recombinant retroviral vector DNA and Lipofectamine 2000 in a 35 mm well. Twenty-four hours post-transfection the PA317 cells were split and selected in hygromycin containing media (100 μg/ml) for 10 days. Mass cultures were prepared from approximately 50–100 colonies and used to produce retroviral vectors stocks. At this point, viral titers were high enough to use for retroviral infection of HC11 Tet-off cells. The HC11-Tet-off cell line was infected with pREV-TRE, pREV-TRE-RasV12, and pREV-TRE-DNRasN17 vector stocks. Cells were selected in hygromycin (100 μg/ml) and doxycyclin (2 μg/ml) for 10 days. Six colonies from each HC11 Tet-off infected cell line were isolated and expanded into cell lines. The clonal cell lines were tested for expression of vector encoded Ras RNA by Northern blot following the removal of doxycyclin.

Adenovirus infection

HC11 and HC11-luci cells were infected with replication defective adenoviruses. A control vector encoding on β-galactosidase (pAd-CMV-β-gal) or a vector encoding Ha-Ras N17, kindly provided by Dr. Craig Logsdon, were used for these experiments (Nicke et al., 1999). Cells were infected with 10–50 MOI of cesium chloride gradient-purified adenovirus by incubation of cells in a low volume of virus-containing media for 5–6 h. The virus was removed and media was added to the cells for 24 h prior to additional treatment of the cells.

Luciferase assays

HC11 luci cells were induced to differentiate in DIP-induction media with and without EGF (10 ng/ml). Inhibitors were added at the time of DIP-induction. Inhibitors were added at optimal concentrations (PD98059, 20 μM; LY294002, 10 μM; wortmannin, 100 nM; SB203580, 10 μM; and tyrphostin A25, 100 μM) determined by dose–response curves (data not shown). Cell lysates were harvested 48 h after transfer to DIP-induction media and luciferase activity was determined using a commercial kit (Luciferase Assay System, Promega) and a luminometer (Thermolab Systems, Ascent, FL). The total cell protein was determined by BCA assay (Pierce[Q4]) and luciferase activity was normalized to protein for all the samples. Results are presented as fold induction calculated from the mean of six determinations.

Electrophoretic mobility shift assay (EMSA)

HC11 cells were grown to confluency in media containing 10% fetal calf serum, 10 ng/ml EGF, and 5 μg/ml insulin then maintained for 3 days without EGF. The cells were then starved for 24 h in serum-free media prior to induction for 15 min and 48 h with DIP as described above. Nuclear extracts were prepared according to a previously published protocol with little modification (Wartmann et al., 1996). Briefly, harvested cells were suspended in CEB (10 mM KCl, 20 mM HEPES, pH 7.0, 1 mM $MgCl_2$, 0.1% Triton X-100, 20% glycerol, 0.1 mM EGTA, 0.5 mM dithiothreitol, 2 mM sodium orthovanadate, 50 μM β-glycerophosphate, 50 mM sodium fluoride, 2 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin) and sheared with 20 strokes using a Dounce homogenizer (Wheaton, pestle B). Nuclei were pelleted by centrifugation at 800*g* for 5 min and then extracted with NEB (CEB + 300 mM NaCl) by incubating for 30 min on ice. Extracts were clarified by centrifugation for 5 min at 16,000*g*. EMSAs were performed by incubating 10 μg of nuclear protein with the Stat5 DNA binding site from the bovine β-casein promoter (5′-AGATTTCTAG-GAATTCAATCC-3′) or Sp1-binding oligonucleotide, end-labeled with ^{32}P-γ-ATP, for 30 min on ice in 16 μl of EMSA buffer (10 mM HEPES, pH 7.6, 2 mM NaH_2PO_4, 0.25 mM EDTA, 1 mM dithiothreitol, 5 mM $MgCl_2$, 80 mM KCl, 2% glycerol, and 100 μg/ml poly [dI-dC]). Specific binding was analyzed on 6% DNA retardation gel and pre-run for 2 h at 200 V in 0.25 × TBE (22.5 mM Tris borate, pH 8.0, 0.5 mM EDTA) at 4°C. The samples were loaded and electrophoresed for 2 h at 200 V, the gels were dried and autoradiographed. For antibody supershift assays, nuclear extracts were pre-incubated with Stat5b C17 antibody (Santa Cruz) for 20 min prior to the addition of the labeled probe.

Northern blots

Total RNA was extracted using TriPure reagent (Roche). Northern blots were prepared using 7.5 or 10 μg of total RNA separated on 1% agarose-formaldehyde gel and transferred to a nylon filter. Blots were hybridized as described previously (Masuelli et al., 1999). The probes used included: mouse β-casein, human KiRas2B, and mouse actin. Mouse β-casein probe is a 601 bp fragment (nucleotide 3–603) from the mouse β-casein cDNA, (accession number X04490.1); it was obtained by RT-PCR and TA-cloning into PCR2.1 and sequence verified. The Ki-Ras probe is a 650 bp fragment representing the human Ki-Ras 2b cDNA, and the actin probe was obtained from Clontech. Mouse Socs-3 probe consisted of nucleotides 467-1006 (accession number NM_007707.2) and mouse Cis-1 was nucleotides 526-1046 (accession number NM_009895.2).

MTT assay

The rate of replication of HC11-TRE and HC11-DNRasN17(12) cell lines was determined by proliferation assay using MTT dye (CellTiter96 Assay by Promega). The cells were propagated for 96 h in the absence of doxycycline. The viable cells were counted by 0.4% trypan blue dye exclusion test and the cell count was adjusted of 1×10^6 cells/ml in RPMI with 0.5% FBS. Cells were plated at density of 1.5×10^3 per well in quadruplicate wells in 96-well plate with or without EGF (10 ng/ml) incubated at 37°C for 24, 48, or 72 h. For analysis of proliferation 15 μl of MTT dye solution was added to each well and the culture plate was incubate at 37°C in CO_2 incubator for 4 h. After 4 h 100 μl of solubilization-stop solution was added to each well. Following 1-h incubation at 37° C the samples were mixed by pipeting and the optical density was measured at 570 nm. The mean and standard deviation of the absorbance values for the quadruplicate wells were calculated.

Immunoprecipitations and Western blots

HC11 cell lysates were prepared in triton-glycerol buffer (1% Triton-X 100, 10% glycerol, 25 mM HEPES, 150 mM NaCl, 2 mM EDTA), NP40 buffer (1% NP40, 25 mM HEPES, 150 mM NaCl) or high salt buffer (Wyszomierski et al., 1999). All lysis buffers contained AEBSF (20 μg/ml), aprotinin, (5 μg/ml), leupeptin, (5 μg/ml), β-glycerol phosphate (100 μM), and $NaVaO_4$ (1 mM). Immunoprecipitates were prepared by incubation of 0.5 or 1 μg of primary antibody with equal amounts of protein (400 μg) and collected by binding to Protein A agarose (Invitrogen[Q5]). Antibodies include anti-Stat5, sc-835 (SantaCruz[Q6]), anti-phosphoStat5 (Cell Signaling[Q7]). For Western blots equal amount of protein were separated by SDS–PAGE and transferred to PVDF filters. Filters were blocked with 2.5% nonfat dried milk (Blotto) in TBS-T for 1 h, then incubated with the appropriate dilution of antibody for 1 h at room temperature or 16 h at 4°C with agitation. Following the incubation with HRP-labeled secondary antibodies, blots were washed and reactivity was detected using ECL (Amersham). Blots were either exposed to Kodak XAR film or results were quantified using a CCD camera (Fuji). Films were quantitated by densitometry. Antibodies included anti-Stat5, sc-835 (SantaCruz), anti-phosphoStat5 (Cell Signaling), anti-phospho Erk, V8031 (Promega), anti-pan Erk (Transduction), anti-AKT and anti-phosphoAKT-ser 473 (Cell Signaling), anti-SHP2 (Transduction), anti-Mek1,2 (Transduction). Anti-PTP-PEST was supplied by Dr. Michael Schaller. Antibodies purchased from Santa Cruz Biotechnology were used at 1 μg/ml, and the antibodies from other suppliers were used at the dilution suggested by the manufacturer.

RESULTS

EGF blocks hormone-induced HC11 differentiation through Mek and PI-3-kinase-dependent pathways

Previous studies demonstrated that EGF blocked lactogenic hormone-induced differentiation of HC11 cells (Hynes et al., 1990), and recent data suggested that this block required Ras and PI-3-kinase activity (DeSantis et al., 1997). In the present study specific chemical inhibitors of signal transduction pathways were used to further analyze the contribution of individual signaling pathways to the block of HC11 differentiation by EGF. Because lactogenic hormone-induced differentiation of HC11 cells is characterized by the initiation of β-casein transcription, the HC11-luci cell line, which contains a β-casein promotor linked to the luciferase gene, was used to provide a rapid readout of the differentiation process.

The HC11-luci cells were induced to differentiate with DIP in the absence and presence of EGF. Specific inhibitors of Mek, and PI-3-kinase were added to cells at the time of induction of differentiation. As expected there was a significant inhibition of β-casein driven luciferase activity in the EGF-treated samples compared to the DIP control. However, several compounds (PD98059, LY294002, and wortmannin) restored the β-casein promotor driven luciferase activity that was blocked by EGF (Fig. 1A). The results demonstrated that the inhibition of Mek–Erk signaling by PD98059 and PI-3-kinase signaling by LY294002 and wortmannin disrupted the EGF signaling that inhibited lactogenic hormone-induced differentiation, as measured by the activation of β-casein promotor driven luciferase expression.

The effect of chemical inhibitors of signal transduction pathways on the synthesis of β-casein RNA was examined (Fig. 1B). The results confirmed that exposure of HC11 cells to DIP activated β-casein expression and that EGF reduced the expression. The inclusion of PI-3-kinase or Mek1 inhibitors in the induction media with EGF reversed the EGF-induced inhibition of the endogenous β-casein promotor activity in the HC11-luci cells.

Previous studies demonstrated that the treatment of HC11 cells with DIP resulted in increased Stat5 DNA binding and that the DNA binding activity of Stat5 was reduced by the simultaneous addition of EGF and lactogenic hormones (Marte et al., 1995). Therefore, EMSA was performed to examine the ability of the signal transduction inhibitors to alter Stat5 DNA binding. Nuclear extracts were prepared from HC11 cells induced to differentiate in the presence of Jak2, Mek1, or PI-3-kinase inhibitors. The results of this reproducible experiment indicated that DIP stimulation in the presence of the Mek1 (PD98059) and PI-3-kinase (wortmannin) inhibitors enhanced Stat5 binding to DNA compared to the binding detected with DIP alone (Fig. 2A). In contrast, exposure of the HC11 cells to DIP plus AG490, an inhibitor of Jak2 tyrosine phosphorylation, inhibited Stat5 DNA binding (Fig. 2A, lanes 4 and 8). The results in Figure 1 indicated that Mek1 and PI-3-kinase inhibitors restored the DIP-induced Stat5 promotor activity inhibited by EGF, and the same Mek and

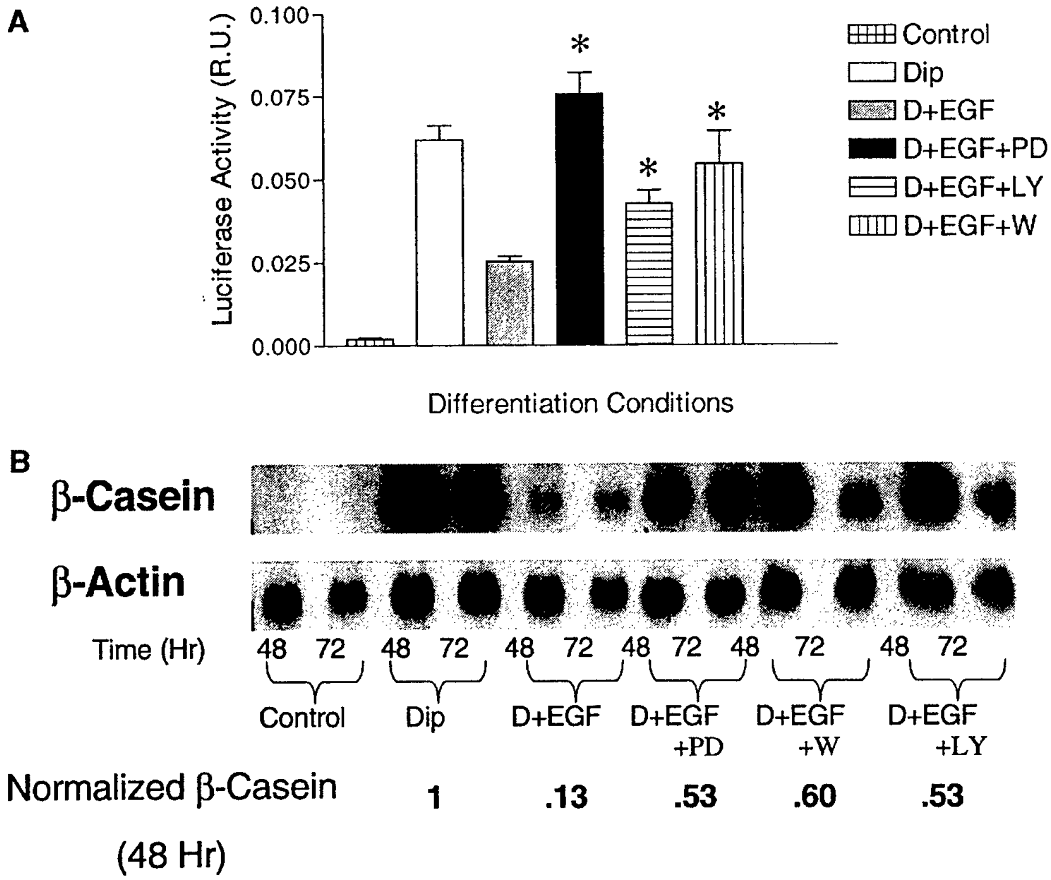


Fig. 1. **A**: The effect of signal transduction inhibitors on epidermal growth factor (EGF) disruption of differentiation. HC11-luci cells were grown to confluence in EGF-containing media then induced to differentiate in DIP-induction media with serum in the presence or the absence of EGF (10 ng/ml). Inhibitors were added at the time of DIP induction at previously determined optimal concentrations (PD98059, 20 μM; LY294002, 10 μM; wortmannin, 100 nM). The luciferase activity in lysates was determined at 48 h post-induction. Luciferase activity was normalized to cell protein. The results, presented as luciferase activity in relative units and represent the mean of six determinations. *, These values represent statistically significant difference (*P* value 0.001) from the DIP + EGF condition. **B**: The effect of signal transduction inhibitors on EGF disruption of β-casein transcription in HC11 cells. The HC11 cells were induced to differentiate in DIP-induction media with and without EGF (10 ng/ml). Inhibitors were added at the time of induction at slightly lower than optimal concentrations to avoid toxicity (PD98059, 10 μM; LY294002, 5 μM; wortmannin, 50 nM). Total cell RNA was harvested at 48 or 72 h after transfer to DIP-induction media. β-Casein induction was determined via Northern blot. For quantitation β-casein expression at 48 h was normalized to β-actin. The level of expression in DIP-treated cells was set as 1.

PI-3-kinase inhibitors enhanced Stat5 DNA binding. Blocking the Mek–Erk and PI-3-kinase pathways with specific inhibitors both enhanced HC11 markers of differentiation and prevented the EGF-dependent disruption of HC11 differentiation.

HC11 cells expressing dominant negative RasN17 exhibit an enhanced lactogenic differentiation response

Because Ras activation regulates the activation of the Erk pathway by EGF and may contribute to the activation of PI-3-kinase, the role of Ras activation in the disruption of HC11 differentiation by EGF was examined. HC11 cell clones expressing either activated Ki-Ras (RasV12) or dominant negative Ki-Ras (DNRasN17) were constructed as described in Materials and Methods. The HC11 cell lines constructed contained the Ras cDNAs under the control of a Tet-responsive promotor in a Tet-off system. Hence, the expression of Ras increased following the removal of doxycycline from the culture media. Several independent clones containing each vector were isolated and characterized for the inducibility of Ras gene expression following the removal of doxycycline from the cultures. As expected, the inducibility varied for the individual RasV12 and DNRasN17 clones. The results obtained with three independent clones derived from each vector are shown in Figure 3.

The DNRasN17 and the RasV12 HC11 cell lines were compared to the vector control cell line, REV-TRE, to determine the effect of the Ras gene expression on lactogenic hormone-induced differentiation. HC11 transfectant cell lines expressing DNRasN17 or activated RasV12 along with the vector control cell line were grown for 72 h in the absence of doxycycline at which point the confluent cultures were incubated in DIP differentiation media. RNA was harvested from cells at 0, 48, 72, and 96 h post-addition of DIP and used to

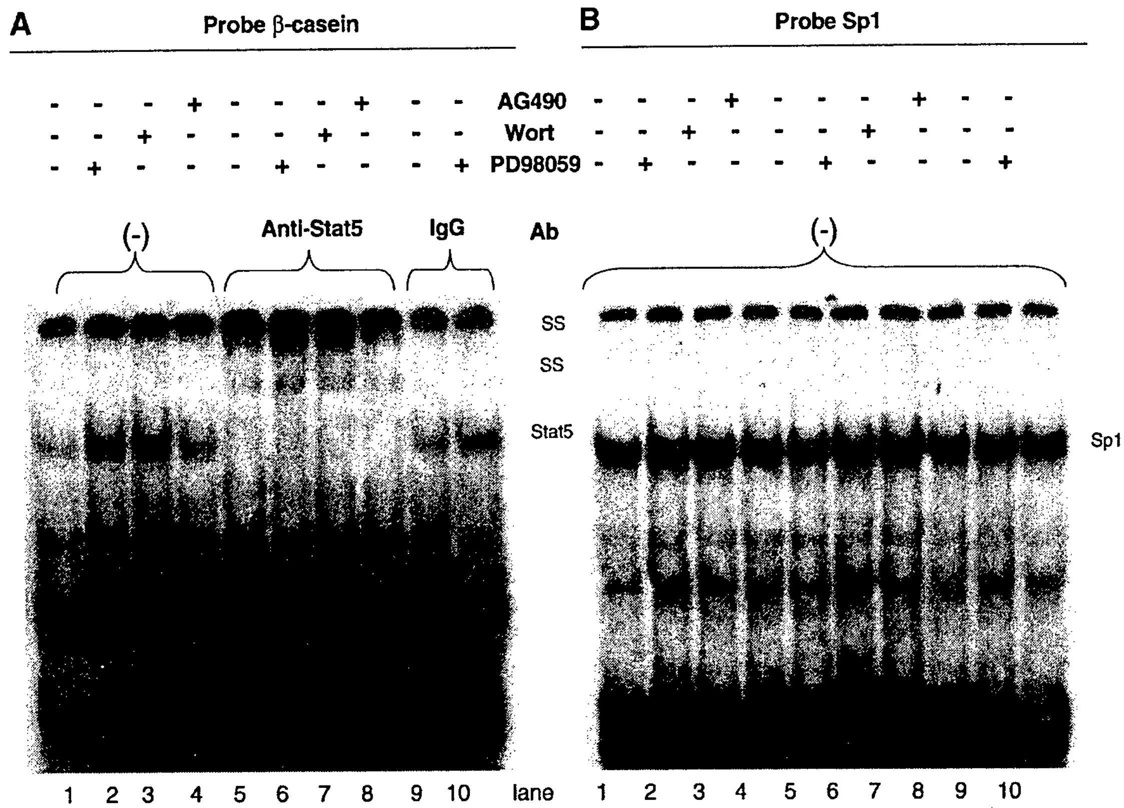


Fig. 2. The effect of inhibitors on Stat5 DNA binding by EMSA. **A:** HC11 cells were grown to confluence in EGF-containing media, then incubated in EGF-free media for 3 days and serum-free media for 1 day. HC11 cells were pretreated with specific kinase inhibitors for 2 h prior to DIP-induced differentiation for 15 min in the presence of the inhibitors. Nuclear lysates were prepared and used for Stat5 binding to the β-casein GAS element in the presence or absence of anti-Stat5 antibody. Lanes 1 and 5: Control (DIP alone); (lanes 2 and 6) PD 98059 (20 μM) plus DIP; (lanes 3 and 7) wortmannin (20 nM) plus DIP; (lanes 4 and 8) AG 490 (20 μM) plus DIP. Lanes 5–8: The binding was performed in the presence of anti-Stat5 antibody for supershift. Lane 9, 10: The samples were the same as (lanes 1, 2) but rabbit IgG was added. **B:** Gel shift (control) using Sp1 oligos as a loading control. The same protein lysates were used as in Part A, but the binding was to an Sp1 oligonucleotide. SS, supershift of Stat5.

determine the level of Ras and β casein expression by Northern blotting. The results in Figure 3 indicated that RasV12 expression inhibited β-casein expression by approximately 50% compared to the TRE control cell line. In contrast, the expression of DNRasN17 enhanced β-casein induction up to twofold compared to the control. The results demonstrated that the amount of DNRasN17 expression correlated with the effect on differentiation. The HC11 cell clone expressing the greatest amount of DNRasN17 (clone 12) exhibited the greatest level of β-casein expression. In contrast, all clones of expressing RasV12 inhibited β-casein expression.

The effect of Ras expression on mammosphere formation, a phenotypic measure of differentiation for primary mammary epithelial cells as well as HC11 cells, was determined. Following growth in the absence of doxycycline, EGF was removed from the cells and lactogenic differentiation was induced by the addition of DIP. The cells were photographed at 0, 72, and 120 h post-DIP and the number of domed mammospheres that appeared in each culture were enumerated (Fig. 4A). At 72 h after DIP addition, the mammospheres were easily counted, but by 120 h the size and the number in the DNRas cell line were too great to count. The results indicated that mammosphere formation was inhibited by RasV12 expression and was significantly enhanced by DNRas expression.

In parallel experiments, the effect of Ras expression on the prolactin-induced tyrosine phosphorylation of Stat5 was examined. HC11 TRE vector control cells as well as the RasV12 (clone 1) and DNRasN17 (clone 12) cells were stimulated with DIP, and the phosphorylation status of the Stat5 protein was determined by immunoprecipitation and Western blotting using anti-Stat5 tyrosine 694 (Y694) phosphorylation site-specific antibodies. The results, seen in Figure 4B, indicated that the tyrosine phosphorylation of Stat5 was enhanced and sustained in the DNRasN17 HC11 cell line compared to the TRE vector control cell line. However, the tyrosine 694 phosphorylation was of a shorter duration in the cell lines expressing activated RasV12 than in the TRE control cells. These results suggested that Ras-dependent signal transduction can modulate Stat5 phosphorylation in HC11 cells in response to DIP. The Stat5 EMSA results supported this conclusion (Fig. 4C). Enhanced Stat5 DNA binding in response to DIP stimulation was observed in the DNRasN17 HC11 cell lysates as compared to the vector control. In contrast, the Stat5 DNA binding activity was reduced in cells

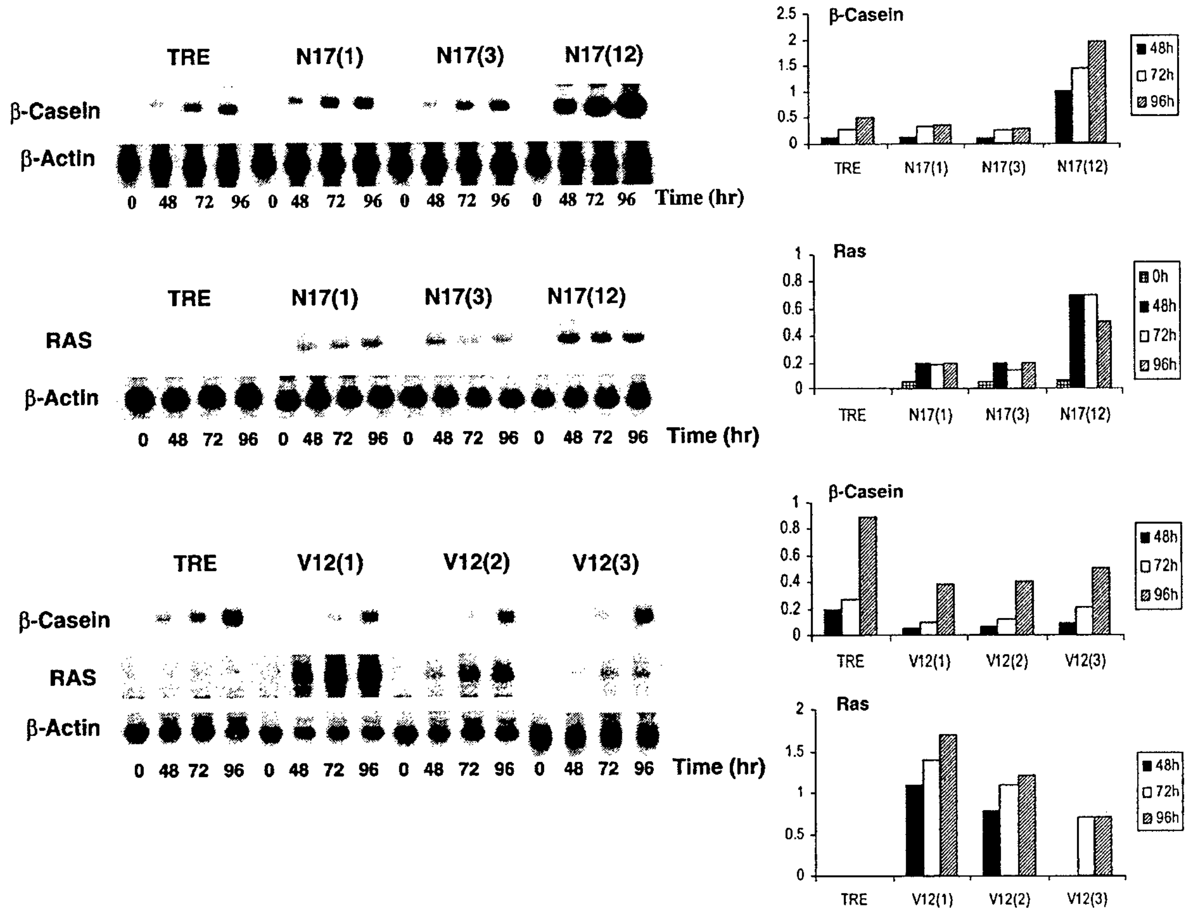


Fig. 3. The effect of RasV12 and DNRasN17 on lactogenic differentiation. HC11 cells expressing activated RasV12 and DNRasN17 under the control of the Tet-responsive promotor were utilized to evaluate the effect of Ras-based signal transduction on lactogenic differentiation. Three individual clones of HC11 cells expressing either RasV12 (clones 1–3) or RasN17 (clones 1, 3, 12) under the control of the Tet responsive promotor were grown to confluence, incubated in the absence of doxycycline and exposed to DIP differentiation media. The vector control cell line, TRE, was treated in parallel. RNA was harvested from cells at 0, 48, 72, and 96 h after addition of DIP and used to determine the level of Ras and β-casein expression by Northern blotting. The Ras and β-casein expression was quantitated using a beta scanner and were normalized to the actin signal and reported in relative units.

expressing activated RasV12. In conclusion, an increase in HC11 cell lactogenic hormone-induced differentiation is observed following blockade of the Ras signaling pathway. Moreover, in the HC11 cells that have Ras activity blocked, the enhancement of hormone-induced differentiation appeared to be attributable to an increase in Stat5 tyrosine phosphorylation and to an increase in Stat5 DNA binding resulting in enhanced transcription of β-casein, a Stat5-regulated gene.

Infection of HC11 cells with dominant negative Ha-Ras adenovirus enhances lactogenic differentiation

Infection of cells with replication defective adenovirus encoding dominant negative Ha-RasN17 (DNRasN17) was used as another mechanism to examine the influence of the Ras pathway on lactogenic differentiation. HC11 cells and HC11-luci cells were infected with 10 MOI of either replication defective control adenovirus or adenovirus encoding DNRasN17. At 48 h post-infection, the cells were examined for the effect of DNRasN17 on Stat5 phosphorylation, β-casein promotor activity and β-casein RNA levels. As demonstrated in Figure 5A HC11-luci cells infected with control virus or DNRasN17 virus were stimulated with DIP and the level of Stat5 tyrosine 694 phosphorylation was determined. The results indicated that the expression of DNRasN17 increased the level of Stat5 phosphorylation in response to DIP compared to either uninfected or vector control-infected cells. HC11-luci cells infected with either replication defective control adenovirus or adenovirus encoding DNRasN17 were tested for activation of β-casein promotor-driven luciferase activity (Fig. 5B). There was a fivefold increase in the activation of luciferase activity in the DNRasN17 cells compared to the uninfected cells or the control adenovirus infected cells. In addition, there was some activation of luciferase activity in cells infected with the DNRasN17 virus without DIP exposure. This result was reproducible and is not seen when uninfected cells or vector infected cells

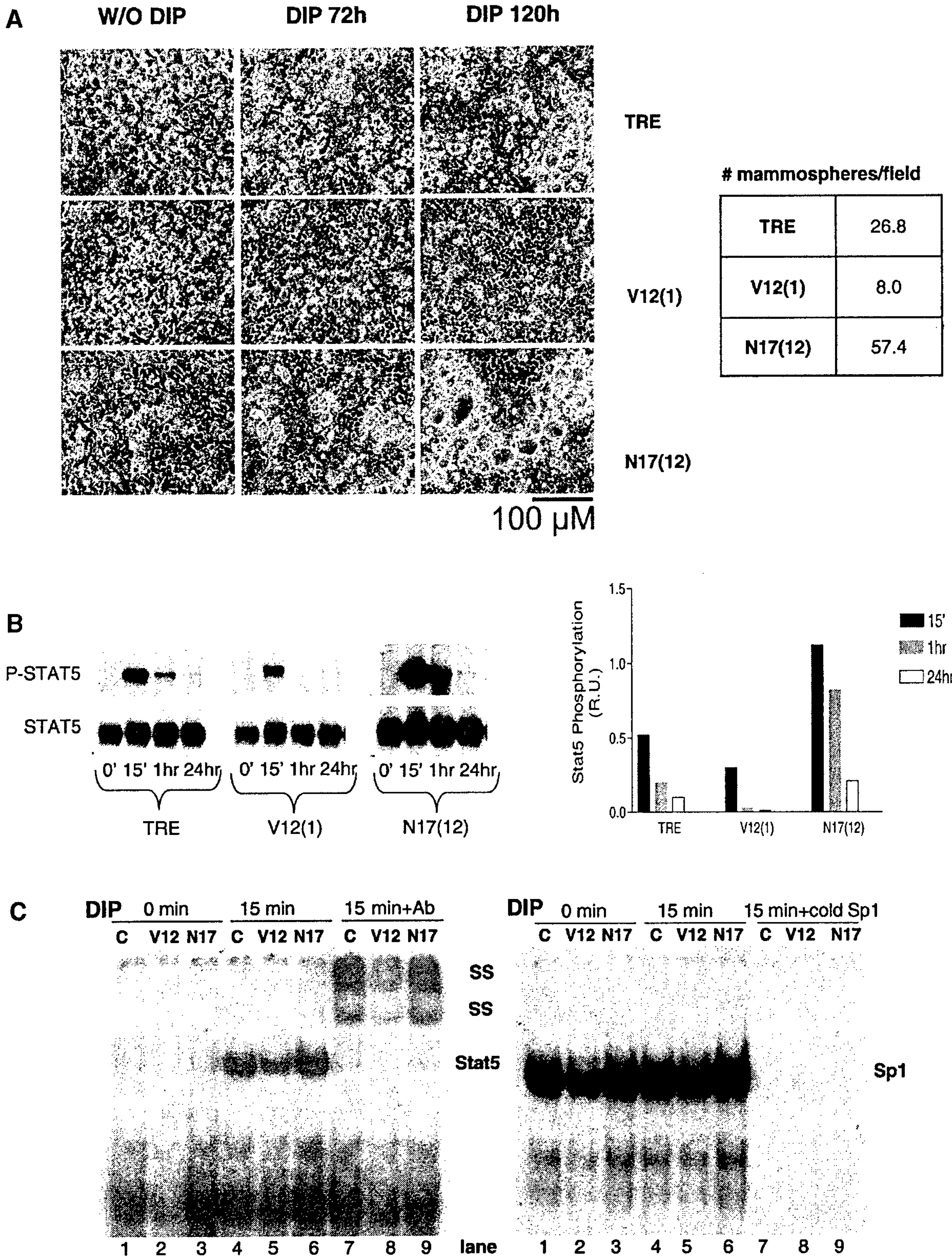

A
W/O DIP
DIP 72h
DIP 120h
TRE
V12(1)
N17(12)
100 μM
mammospheres/field
TRE 26.8
V12(1) 8.0
N17(12) 57.4
B
P-STAT5
STAT5
0' 15' 1hr 24hr
TRE
V12(1)
N17(12)
Stat5 Phosphorylation (R.U.)
1.5
1.0
0.5
0.0
15'
1hr
24hr
C
DIP
0 min
15 min
15 min+Ab
C V12 N17
SS
SS
Stat5
15 min+cold Sp1
Sp1
lane
1 2 3 4 5 6 7 8 9

Figure 4

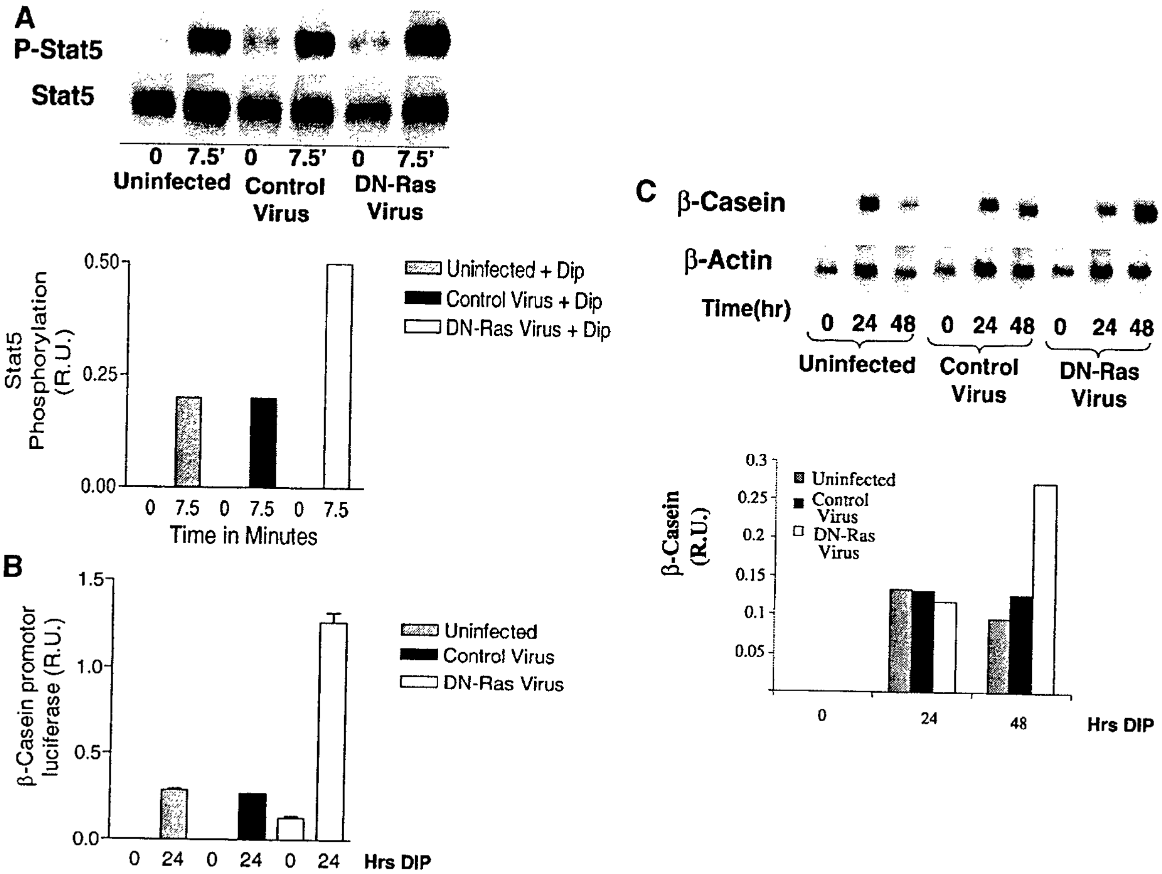


Fig. 5. The effect of dominant negative Ha-Ras (N17) adenovirus expression on lactogenic differentiation in HC11 cells. **A**: The effect of DNRasN17 adenovirus on Stat5 phosphorylation in response to lactogenic hormone was determined. Uninfected HC11 cells, HC11 cells infected with a control adenovirus vector and HC11 cells infected with adenovirus encoding DNRas (N17) (at MOI = 10) were incubated for 24 h; the cells were then serum-starved overnight and stimulated with DIP for 7.5 min. Total Stat5 was immunoprecipitated and analyzed by Western blotting with antibodies for phospho-Stat5 or total Stat5. The amount of phospho-Stat5 and total Stat5 was quantitated using a CCD camera, and the amount of phospho-Stat5 was normalized to the total Stat5 and reported in relative units. **B**: HC11-luci cells infected with adenovirus vector control or adenovirus encoding dominant negative RasN17 were used to determine the effect of DNRasN17 on β-casein driven luciferase activity. The cells were infected with the viruses described above and incubated for a period of 24 h in media without EGF. The cells were then either stimulated with DIP for 24 h or incubated in media without EGF for an additional 24 h. The luciferase activity in lysates was determined and normalized to cell protein; the results, presented as luciferase activity in relative units, represent the mean of four determinations. **C**: The effect of DNRasN17 adenovirus infection on HC11 expression of β-casein was determined. The HC11 cells were infected with the control or DNRasN17 virus as described above. RNA was isolated at 0, 24, and 48 h post-induction of differentiation and used to determine the amount of β-casein transcription by Northern blotting. Hybridization of the blots with an actin probe was used as a control for RNA loading. The expression of the β-casein RNA was quantitated by measurement on a β-scanner, normalized to actin and expressed on a relative scale.

Fig. 4. The effect of RasV12 and DNRasN17 expression on mammosphere formation, Stat5 phosphorylation and DNA binding. **A**: HC11 TRE vector control cells and HC11 cell lines expressing activated RasV12 (clone 1) or DNRasN17 (clone 12) were grown to confluence and exposed to DIP as described in Materials and Methods. The cells were photographed at 0, 72, and 120 h post-DIP addition. The number of mammospheres per field is reported; this was determined by counting the number of mammospheres per low power field and determining the mean of five fields. **B**: HC11 TRE vector control cells and HC11 cell lines expressing activated RasV12 (clone 1) or DNRasN17 (clone 12) were grown to confluence in EGF-containing media without doxycycline to induce the expression of Ras. The cells were stimulated with DIP, and nuclear extracts were prepared from cells at 0, 15 min, 1 and 24 h post-stimulation. Total Stat5 was immuno-precipitated and analyzed by Western blotting with antibodies for phospho-Stat5 or total Stat5. The amount of phospho-Stat5 and total Stat5 on the Western blots was quantitated, and the amount of phospho-Stat5 was normalized to the total Stat5 and reported in relative units. **C**: EMSA. HC11 TRE vector control, RasV12(1), and RasN17(12) cells were grown to confluence in EGF-containing media, then incubated in EGF-free media for 3 days and serum-free media for 1 day. Treated cells were exposed to differentiation media for 15 min and control cells (T = 0) were not exposed to DIP. Left part[Q8]: Nuclear lysates were prepared and used for Stat5 binding to the β-casein GAS element in the presence or absence of anti-Stat5 antibody as indicated. Lanes 1, 4, 7: TRE control; (lanes 2, 5, 8) RasV12 (1); (lanes 3, 6, 9), RasN17(12). Right part: Sp1 binding oligonucleotides were used as a loading control. Lanes 1, 4, 7: TRE control; (lanes 2, 5, 8) RasV12(1); (lanes 3, 6, 9) RasN17(12). Lanes 1–3: Contain control lysate; (lanes 4–6) contain lysate from DIP-treated cells; (lanes 7–9) contain lysate from DIP-treated cells with the addition of 50 × cold Sp1 oligonucleotides. SS: Stat5 supershift.

were exposed to DIP. Finally, HC11 cells infected with either replication defective control adenovirus or adenovirus encoding DNRasN17 were examined for expression of the endogenous β-casein gene following exposure to DIP for 24 or 48 h. The results of Northern blots (Fig. 5C) indicated that the infection with DNRasN17 virus resulted in a twofold increase in β-casein RNA compared to the uninfected or vector infected cells exposed to DIP.

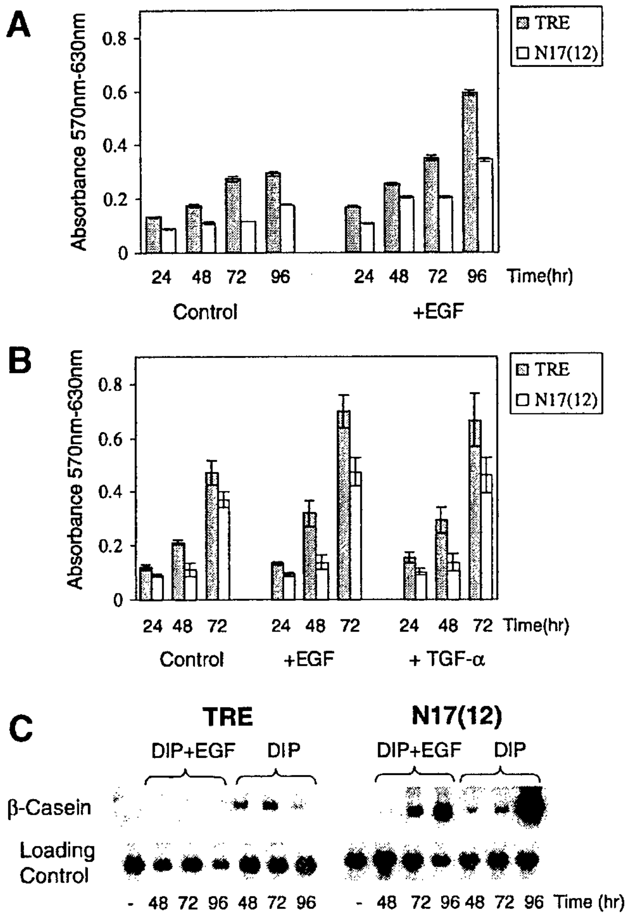
HC11 cells expressing dominant negative Ras exhibit reduced response to EGF

Studies were performed to determine if the DNRasN17 expression could block EGF-induced responses in stable transfectants of HC11 cells. HC11 cells respond mitogenically to EGF. The TRE vector control cells and the DNRasN17 cells were stimulated with EGF and the ability of the cells to proliferate was examined using the MTT assay. Cells were removed from doxycylcine for 96 h and then grown in reduced serum media in the absence and the presence of EGF. MTT assays were performed over the course of 4 days to follow cell proliferation. The results in Figure 6A demonstrated that the DNRasN17 cell line was growth inhibited by 40% in both the absence and presence of EGF compared to the vector control cell line. This experiment was repeated using TGFα treatment of HC11 vector control and DNRasN17 cells. Again, the DNRasN17 cells exhibited a significantly lower response to EGF and TGFα than did the vector control cell line (Fig. 6B).

The ability of DNRas to prevent the disruption of lactogenic hormone-induced differentiation by EGF in HC11 cells was examined. The HC11 TRE vector control cells and cells expressing DNRasN17 under the control of a Tet-responsive promotor were grown in the absence of doxycycline for 72 h. The cells were exposed to lactogenic hormone differentiation media in the presence and absence of EGF for varying lengths of time; RNA was extracted and the level of β-casein mRNA was analyzed by Northern blotting. The results in Figure 6C demonstrated that EGF did not inhibit the induction of β-casein transcription in the DNRasN17 cell line and, hence, it appeared that differentiation proceeded in these cells even in the presence of EGF. In contrast, the expression of β-casein was blocked by EGF in the TRE vector control cell line in two separate experiments. These results demonstrated that DNRasN17 expression prevented the disruption of hormone-induced differentiation by EGF in HC11 cells.

HC11 cells expressing dominant negative Ras exhibit reduced Erk activation in response to EGF

HC11 cells expressing DNRasN17 were examined to determine if expression of DN Ras prevented the activation of Mek–Erk or PI-3-kinase signaling in response to EGF. In Figure 7A the stable transfectants were removed from doxycycline and grown to confluence. The cells were starved and then stimulated with EGF for varying amounts of time. Cell lysates were prepared and analyzed by Western blot using antibodies that detect phosphorylated forms of different signaling proteins. The results, shown in Figure 7A, revealed that stimulation of HC11 vector control cells with EGF resulted in activation of p44Erk as detected by reactivity



Experiment #	Cell Line	% Reduction β-Casein RNA
1	TRE	99.1 %
	N17(12)	0 %
2	TRE	41.5 %
	N17(12)	0 %

Fig. 6. DNRas N17 expression inhibits EGF-induced proliferation and prevents EGF-dependent disruption of lactogenic differentiation. **A**: HC11 TRE vector control and DNRasN17 (clone 12) cells were grown in absence of doxycycline and then seeded in microtiter plates in 0.5% serum-containing media with and without EGF (10 μg/ml). Cell proliferation was determined at 24, 48, 72, 96 h post-addition of EGF using the MTT assay. The results are reported as the mean of four determinations. **B**: The HC11 TRE vector control and DNRasN17 (clone 12) cells were grown as described above and exposed to EGF (10 ng/ml) or TGFα (10 ng/ml). Cell proliferation was determined using the MTT assay and the results represent the mean of four determinations. **C**: HC11 TRE vector control and RasN17 (12) cells were grown to confluence in absence of doxycycline and then exposed to DIP in the presence or absence of EGF (10 ng/ml). Total RNA was isolated after 72 h and used for Northern blotting. The blots were hybridized to probes for β-casein and actin. The β-casein and actin RNA was quantitated using a beta scanner; the β-casein RNA was normalized to the actin RNA. The % reduction of β-casein RNA by the addition of EGF during DIP-induced differentiation was calculated using the values for normalized β-casein expression.

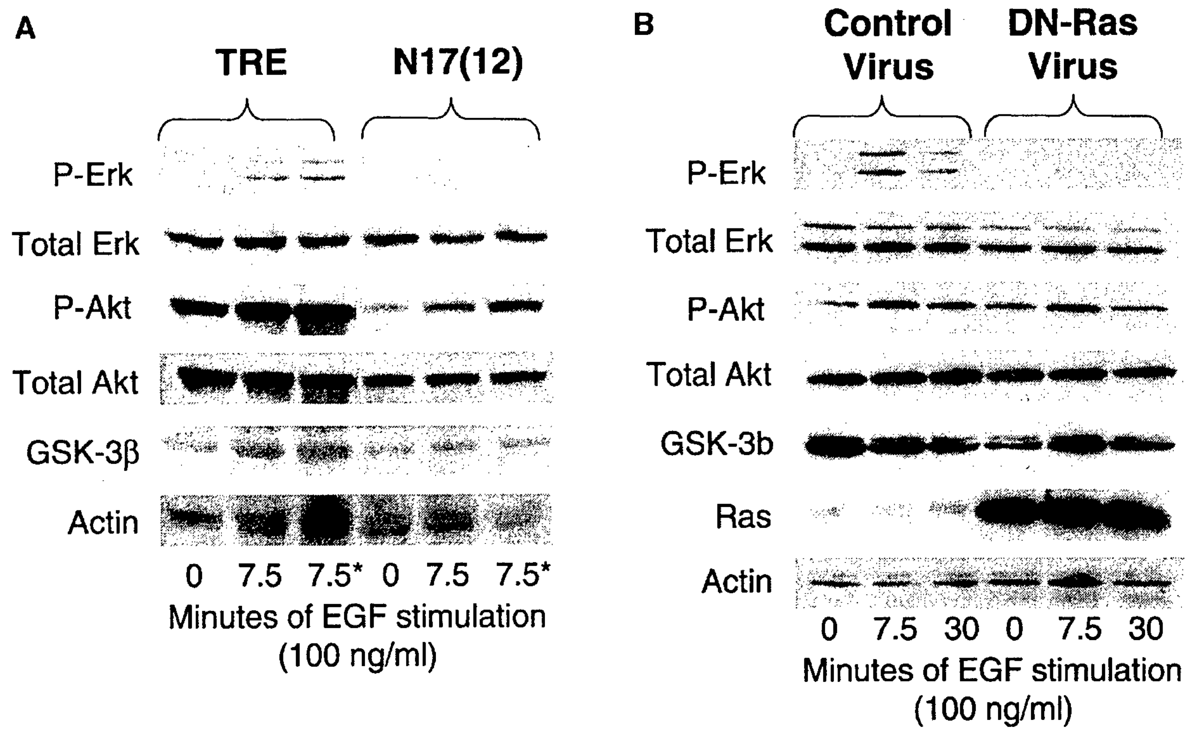


Fig. 7. The effect of DNRasN17 expression on signal transduction pathways in HC11 cells. **A**: The HC11 TRE vector control cells and DNRasN17 (clone 12) cell lines were grown to confluence in EGF-containing media lacking doxycycline. The cells were incubated in media without EGF or media without EGF and serum (*) prior to restimulation with EGF (100 ng/ml) for the time indicated. Lysates of cells were harvested and analyzed by Western blotting using antibodies specific for phosphorylated and nonphosphorylated forms of the indicated proteins. **B**: HC11 cells infected with control adenovirus vector or DNRasN17-encoding adenovirus at an MOI of 10 were incubated in serum-containing media for 24 h and incubated in EGF-free media for 20 h prior to stimulation with EGF (100 ng/ml) for the time indicated. Lysates of cells were harvested and analyzed by Western blotting as in part A.

with an antibody that recognizes the active phosphorylated forms of Erk1, 2. In contrast, in HC11 cells expressing DNRasN17 there was no activation of p44Erk, although the Erk protein levels in the cells were similar to those in the vector control cells. The analysis of other signaling proteins revealed little or no difference in Akt activation between the control HC11 cells and the DNRasN17 HC11 cells following treatment with EGF. This demonstrated that the PI-3-kinase pathway was not significantly blocked by DNRasN17 expression in HC11 cells. Moreover, activation of Jun kinase and p38 kinase by EGF was not deficient in the DNRasN17 HC11 cells (data not shown). These results suggest that the Mek–Erk pathway was most sensitive to inhibition by DNRas17 expression.

Cells infected with the control adenovirus vector or adenovirus encoding DNRasN17 were examined for the effect of EGF on signal transduction pathways in an analogous fashion. The results in Figure 7B demonstrated that DNRasN17 adenovirus also blocked the activation of Erk but not the phosphorylation of AKT on serine 473, used as a measure of PI-3-kinase activity. The results from the DNRasN17 expressing cells indicated that blocking the Ras pathway in this manner in HC11 cells primarily blocked signaling to the Raf–Mek–Erk pathway. Hence, these data support the conclusion that in HC11 cells activated RasV12 inhibits β-casein transcription via Mek–Erk signaling, and that the effect of DNRasN17 expression on β-casein is primarily a result of its inhibition of the Mek–Erk pathway.

Expression of dominant negative Ras prevents the prolactin-induced association of SHP2 with STAT5

Our results demonstrated that the expression of DNRasN17 resulted in enhanced DIP-induced activation of STAT5 as measured by tyrosine phosphorylation, DNA binding and activation of the β-casein promotor. To determine the mechanism by which this occurs, the functionality of several STAT5 regulatory pathways in DNRasN17 cells was examined. The increased activity of STAT5 likely resulted from the higher level of STAT5 tyrosine 694 phosphorylation; hence, regulation of STAT5 tyrosine phosphorylation was examined by looking for the association of tyrosine phosphatases with Jak2 and STAT5. Previous reports demonstrated that the phosphatase SHP2 interacted with STAT5; SHP2 associated with STAT5 following stimulation of the STAT5 pathway by prolactin as detected by co-immunoprecipitation of SHP2 with STAT5 (Chughtai et al., 2002). To determine if dominant negative Ras expression affected the prolactin-induced association of SHP2 with STAT5, HC11-TRE, and DNRasN17 cells were stimulated with prolactin. Cell lysates were prepared, STAT5 was immunoprecipitated, and the amount of SHP2 associated with the STAT5 was determined by

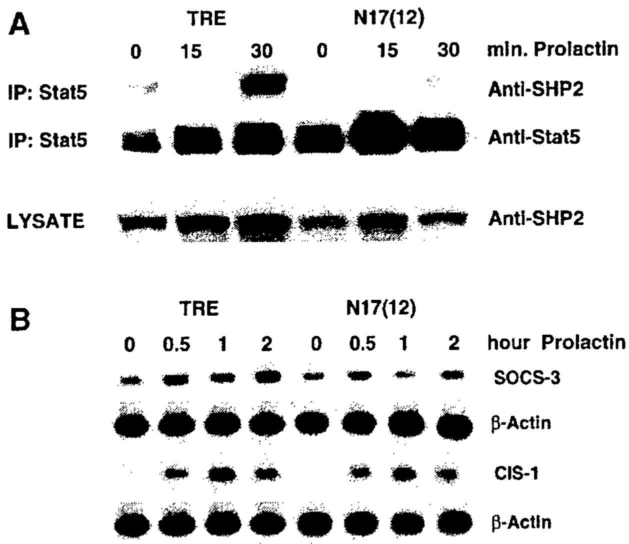


Fig. 8. Prolactin-induced binding of SHP2 to Stat5 and prolactin-induced expression of SOCS-3 and CIS-1. HC11-TRE and HC11-DNRasN17 (clone 12) cells were grown to confluency in EGF-containing media then incubated in EGF-free media for 24 h; the cells stimulated with prolactin (5 μg/ml) for the indicated time. **A**: Lysates were prepared and Stat5 was immunoprecipitated. The lysates and the immunoprecipitates were analyzed by Western blotting for Stat5 and SHP2. **B**: RNA was extracted from cells and analyzed by Northern blotting for expression of SOCS-3 and CIS-1. β-Actin was hybridized to the blots as a loading control.

Western blotting. The results in Figure 8A demonstrated that the level of SHP2 protein expression was not reduced in HC11-DNRasN17 cells. However, in the vector control cell line, prolactin stimulation for 30 min resulted in significant association of SHP2 with STAT5, but very little SHP2 was associated with STAT5 in prolactin stimulated DNRasN17 cells (Fig. 8A). Accordingly, the degree of STAT5 phosphorylation at Y694 was significantly greater in the DNRasN17 cells than in the TRE cells.

In previous reports PTP-PEST was identified as a phosphatase that was activated by EGF and prolactin and associated with Jak2 (Horsch et al., 2001). These studies indicated that PTP-PEST was co-immunoprecipitated with JAK-2 from HC11 cells following prolactin stimulation. However, we did not observe this association in either the HC11-TRE or HC11-DNRasN17 cell lines (data not shown).

A previous report indicated that both EGF and prolactin stimulation induced the expression of the inhibitors of cytokine signaling, SOCS-3 and CIS-1, in HC11 cells (Tonko-Geymayer et al., 2002). To determine if changes in the regulation of SOCS-3 and CIS-1 expression was affected by dominant negative Ras expression, the level of SOCS-3 and CIS-1 expression was examined by Northern blotting at various times after the addition of prolactin to HC11-TRE and DNRasN17 cell lines. The results, shown in Figure 8B, indicated that the expression of CIS-1 and SOCS-3 was stimulated by prolactin and that the expression was similarly regulated in the HC11- DNRasN17 cells and the HC11-TRE control cell line.

These results indicated that the association of SHP2 tyrosine phosphatase activity with STAT5 was blocked by dominant negative Ras expression. The resulting block to STAT5 dephosphorylation constitutes a likely mechanism for the enhancement of STAT5 activation by DNRasN17 expression.

DISCUSSION

Members of the EGF family of peptide growth factors are found in the mammary gland and appear to play a role in growth and differentiation in that tissue (Jhappan et al., 1990). For example, EGF and amphiregulin are expressed in the ductal epithelial cells and TGFα is expressed in cap stem cells in the terminal end buds (Snedeker et al., 1992; Kenney et al., 1995). EGF and TGFα, bind to EGF receptor (ERB1) and can stimulate the proliferation of mammary epithelial cells and enhance lobular–aveolar development in the mammary gland of virgin mice (Vonderhaar, 1987). These growth factors can also prevent milk protein expression in HC11 cells and inhibit apoptosis of secretory alveolar epithelial cells in the involuting mammary gland (Smith et al., 1995). Hence, these factors play a dual role in mammary differentiation.

Growth factors of the EGF family have been detected in human breast tissue and elevated levels have been associated with breast tumors (Dotzlaw et al., 1990; Mizukami et al., 1991). The stimulation of mammary cells in culture by these growth factors activates signal transduction pathways that lead to cell survival and mitosis, and the activation of the EGF-R (ErbB1) correlates with aggressive behavior of breast tumors (Arteaga et al., 1988; Umekita et al., 1992). One of the signaling molecules activated by EGF family growth factors in breast tumors is the Ras GTPase (von Lintig et al., 2000). Previous studies have demonstrated that EGF and activated Ras inhibit differentiation in HC11 cells. Both stimulation of mammary epithelial cells with EGF and the expression of activated Ras initiate signaling through the Mek–Erk pathway. While EGF stimulation also leads to activation of the PI-3-kinase pathway, the influence of Ras on this pathway in HC11 cells has not been examined. Therefore, the present study analyzed the effects of EGF on the Ras, Erk, and PI-3-kinase pathways in HC11 cells and the contribution of those pathways to lactogenic hormone-induced differentiation.

The results confirmed the findings of several previous studies by demonstrating that EGF can block lactogenic hormone-induced differentiation in HC11 cells (Hynes et al., 1990). Chemical inhibitor studies indicated that the inhibition of β-casein promotor activity by EGF required both the Mek–Erk and PI-3-kinase pathways. While a previous study found that activation of the Erk pathway was not required for lactogenic differentiation (Wartmann et al., 1996), the contribution of Erk to the inhibition of lactogenic hormone-induced differentiation by EGF was less clear. Merlo et al. (1996) correlated the inhibition of lactogenic hormone-induced differentiation by growth factors with the ability of different growth factors to induce a high level of Erk activation. Also, expression of v-Raf, an activator of Mek–Erk signaling, inhibited lactogenic hormone-induced differentiation of HC11 cells (Happ et al., 1993). However, a previous study by DeSantis et al. (1997) demonstrated that inhibition of Ras and PI-3-kinase blocked the inhibitory effects of EGF on β-casein synthesis. Our study extends this previous study and demonstrates that the inhibition of the Erk pathway strongly correlates with an increase in β-casein promotor activation. Moreover, in our study the stable expression of dominant negative Ki-RasN17 or the infection of HC11 cells with dominant negative Ha-RasN17 adenovirus effectively enhanced β-casein synthesis in response to lactogenic hormones, and these cells exhibited inhibition of the Mek–Erk pathway but not the PI-3-kinase signaling pathway. Hence, it appears that the Erk pathway is critical in the negative regulation of lactogenic hormone-induced differentiation by DNRas. This appears be a function of its effect on Stat5 tyrosine phosphorylation and activation. Our results are in agreement with those of Gao et al., which suggest that Erk activation alters prolactin-induced expression at a step prior to Stat5 DNA binding (Gao and Horseman, 1999). The HC11 cells expressing dominant negative Ras, which were defective in Erk activation, exhibited both an increase in Stat5 tyrosine phosphorylation and an increase in Stat5 DNA binding.

The SH2 protein tyrosine phosphatase, SHP2, has been identified in a complex with Stat5 and a role for this phosphatase in regulation of Stat5 activity has been proposed (Berchtold et al., 1998; Chughtai et al., 2002). Our results indicated that DNRas expression blocked the association of SHP2 with Stat5. The mechanism by which this occurs has not been resolved. In addition to involvement in Jak-Stat signaling, SHP2 is required for growth factor receptor activation of the Ras–Erk pathway. SHP2 plays an essential role in linking components of signal transduction pathways to growth factor receptor complexes via the scaffold protein Gab1, which targets SHP2 to the membrane (Cunnick et al., 2002). Two potential links for SHP2 to the Ras pathway have been reported. Dominant negative SHP2 expression decreased the level of activated Ras (Ras-GTP) in cells (Cai et al., 2002); this could result from a decrease in guanine nucleotide exchange factor (SOS) activity. Alternatively, it was recently reported that SHP2 regulated EGF-dependent RasGAP, but not SOS, membrane localization and increased the half-life of Ras-GTP (Agazie and Hayman, 2003). Our data demonstrated that dominant negative Ras expression, which interferes with Ras activation in part by binding and sequestering guanine nucleotide exchange factors (Lai et al., 1993), disrupts one aspect of SHP2 function. This suggests that there may exist a mechanism to regulate SHP2 by Ras via SOS or RasGAP.

While EGF stimulation of HC11 cells has been linked to the activation of PTP-PEST and dephosphorylation of Jak2 (Horsch et al., 2001), no association of PTP-Pest with Jak2 was detected following prolactin stimulation in the HC11-TRE or HC11-DNRasN17 cell lines. In addition, although expression of SOCS-3 and CIS-1 has been demonstrated in HC11 cells following exposure to prolactin (Tonko-Geymayer et al., 2002), DNRasN17 expression did not alter the transcriptional activation of Socs-3 or Cis-1 following prolactin stimulation in our experiments. Collectively these results suggested that DNRasN17 expression enhanced Stat5 tyrosine phosphorylation primarily by blocking the association of the SHP2 phosphatase with Stat5.

The data presented here demonstrate that the addition of EGF to HC11 cells stimulates the PI-3-kinase pathway resulting in the phosphorylation of Akt and its downstream signaling pathway. The data also demonstrate that inhibition of the PI-3-kinase pathway increases β-casein promotor activity. The expression of dominant negative Ki-Ras did not prevent the activation of PI-3-kinase-Akt pathway, indicating that the activation of PI-3-kinase was primarily a consequence of the binding of the p85 subunit to the EGF receptor rather than the direct activation of p110 by activated Ras (Rodriguez-Vicania et al., 1994). These results suggest that the PI-3-kinase pathway influences a stage in Jak-Stat signaling that occurs prior to or at the level of DNA binding. A recent study has demonstrated that PI-3-kinase inhibition enhanced Stat5 activation by thrombopoitin in part by preventing nuclear export of Stat5 (Kirito et al., 2002).

There have been several studies in other tissues demonstrating that regulation of Ras-dependent signal transduction contributes to differentiation. For example, there is evidence from both in vivo systems and tissue culture systems that the Ras–Raf–Mek–Erk pathway is required for neuronal differentiation (Halegoua et al., 1991; Thomas et al., 1992; Wood et al., 1992; Cowly et al., 1994; Marshall, 1995). Also, the activation of the Mek–Erk pathway may contribute to the differentiation status of some breast cancer cell lines. For example, differentiation-linked Erk activation in breast cancer cells occurs following ligand-induced activation of RTKs, including stimulation with heregulin (NDF, Neu differentiation factor) and subsequent activation of HER-3 (Lessor et al., 1998), or following transfection and overexpression of c-erbB-2 (Giani et al., 1998). In both systems activation of the Ras–Erk pathway resulted in increased expression of $p21^{CIP}$ and enhanced differentiation. ErbB4 signaling has also been linked to prolactin-induced Stat5 activation (Jones et al., 1999). Hence, because of dual nature of Mek–Erk signaling in differentiation, it is important to understand the role of the Ras pathway in lactogenic hormone-induced differentiation. The results of this study clearly focus on signaling through the Mek–Erk pathway as a Ras-regulated disruptor of lactogenic hormone-induced differentiation. Moreover, by identifying the Mek–Erk pathway along with altered regulation of SHP2 as pathways that are inhibited by DNRasN17, these studies suggest an additional mechanism by which EGF disrupts differentiation in this cell line.

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Figure 8. The gene expression pattern in differentiated HC11 cells compared to control undifferentiated HC11 cells were determined using DNA microarray technique. The results were obtained using two different arrays, **A**. Agilent 20K oligonucleotide array and **B**. Clontech 5K cDNAs array. **C**. gene expression in HC11 cells induced to differentiate in the presence versus the absence of EGF.

A

DIP/CON, (Agilent)

mean	p-value	GB acc	UG cluster	Description
17.0692248	0.0000098	NM_010217.1	Mm.1810	connective tissue growth factor
13.4421959	0.0004945	BC027319		Mus musculus ATPase, Na+/K+ transporting, beta 1 polypeptide
11.14873952	0.0002881	XM_150141.2		LOC234574
9.522688101	0.0000609	NM_009263.1	Mm.321	secreted phosphoprotein 1
7.69516999	0.0006805	BC009155.1	Mm.14796	microsomal glutathione S-transferase 1
7.643536442	0.0002742	NM_022032.1	Mm.28209	p53 apoptosis effector related to Pmp22
6.986319761	0.0013017	NM_011326.1	Mm.35247	"sodium channel, nonvoltage-gated 1 gamma"
6.439168301	0.0018506	NM_009681.1	Mm.27171	"adaptor-related protein complex AP-3, sigma 1 subunit"
6.335661401	0.0005149	NM_029083.1	Mm.21697	RIKEN cDNA 5830413E08 gene
5.938412871	0.0013857		Mm.10029	"DNA segment, Chr 15, Brigham & Womens Genetics 0759 expressed"
5.923853709	0.0003607	BC036990.1	Mm.192991	metallothionein 1
5.847013528	0.0028077	XM_123496.1	Mm.24621	Tcfcp2-related transcriptional repressor 1
5.775174223	0.0006173	NM_007621.1	Mm.21454	carbonyl reductase 2
5.773951091	0.001373	NM_138578.1	Mm.3882	Bcl2-like
5.759602371	0.0006522	NM_012519.1	Mm.34377	"calcium/calmodulin-dependent protein kinase II, delta"
5.719155211	0.0004807	NM_009976.1	Mm.4263	cystatin C
5.416841983	0.0001758	NM_010286.1	Mm.22216	glucocorticoid-induced leucine zipper
5.147111567	0.0021434	NM_009128.1	Mm.193096	stearoyl-Coenzyme A desaturase 2
5.14084859	0.000177	NM_025610.1	Mm.20246	RIKEN cDNA 2410004D18 gene
5.134731027	0.0041275	NM_011361.1	Mm.28405	serum/glucocorticoid regulated kinase
0.570000993	0.0309545	NM_025836.1	Mm.104975	RIKEN cDNA 1300012C15 gene
0.56666635	0.0076789		Mm.214924	ESTs
0.565456881	0.0220481		Mm.11987	"ESTs, Moderately similar to POL2_MOUSE Retrovirus-related POL
0.539313291	0.0237038		Mm.173695	"ESTs, Moderately similar to cofactor required for Sp1 transcriptional
0.444009365	0.0185901	NM_008086.1	Mm.22701	growth arrest specific 1
0.377664008	0.0011905	BC012724.1	Mm.141936	insulin-like growth factor binding protein 2

Figure 8 (Continued)

B

DIP/CON (Clontech)

accession	mean	Name
NM_010217	6.02275	connective tissue growth factor
NM_008843	4.232	prolactin induced protein
NM_013467	4.11725	"alcohol dehydrogenase family 1, subfamil"
NM_008489	3.9215	lipopolysaccharide binding protein
NM_008952	3.54625	peroxisomal sarcosine oxidase
NM_013515	3.34025	erythrocyte protein band 7.2
NM_009721	3.318	"ATPase, Na+/K+ transporting, beta 1 poly"
NM_011338	2.9665	small inducible cytokine A9
NM_012046	2.87525	"sporulation protein, meiosis-specific, S"
NM_011461	2.85725	Spi-C transcription factor (Spi-1/PU.1 r
NM_008065	2.6545	"GA repeat binding protein, alpha"
NM_007607	2.54975	carbonic anhydrase 4
NM_011361	2.399	serum/glucocorticoid regulated kinase
NM_019496	2.05575	"Alport syndrome, mental retardation, mid"
NM_009681	1.96225	"adaptor-related protein complex AP-3, si"
NM_009775	1.95425	"benzodiazepine receptor, peripheral"
NM_009779	1.92625	complement component 3a receptor 1
NM_013634	1.849	peroxisome proliferator activated recept
NM_013799	1.8485	arginine-tRNA-protein transferase 1
NM_011057	1.8335	"platelet derived growth factor, B polype"
NM_009127	1.8125	stearoyl-Coenzyme A desaturase 1
NM_007918	1.80025	eukaryotic translation initiation factor
NM_009320	0.599	solute carrier family 6 (neurotransmitte
NM_008536	0.5975	transmembrane 4 superfamily member 1
NM_011271	0.5115	"ribonuclease 1, pancreatic"
NM_008764	0.41725	tumor necrosis factor receptor superfami
NM_010701	0.331	leukocyte cell derived chemotaxin 1

Figure 8 (Continued)

C

(DIP+EGF)/DIP, Agilent

mean	p-value	GB acc	UG cluster	Description
2.629325346	0.0049573	NM_009263.1	Mm.321	secreted phosphoprotein 1
2.585538601	0.0000395	NM_145597.1	Mm.23488	cDNA sequence BC021367
2.54281691	0.0008723	NM_016980.1	Mm.4419	ribosomal protein L5
2.522356968	0.0000113	AF378830.1	Mm.3137	prostaglandin endoperoxide synthase 2
2.456467561	0.0000237	NM_008972.1	Mm.19187	prothymosin alpha
2.347383128	0.0010689	NM_009121.1	Mm.2734	spermidine/spermine N1-acetyl transferase
2.33154908	0.0004548	NM_019682.1	Mm.29908	"dynein, cytoplasmic, light chain 1"
2.318271338	0.0000407	BC006739.1	Mm.3476	catenin beta
2.307858951	0.0000824	NM_133777.1	Mm.22491	RIKEN cDNA 6720465F12 gene
0.500465463	0.000061	AE014180.1		NA
0.495838452	0.0014903	BC033410.1	Mm.141058	eukaryotic translation initiation factor 4E nuclear import factor 1

Figure 9.

<u>Names and abbreviations of genes used for Nothern blot probes</u>

••fold changes	Gb acc	name	abbr.
••Agilent Arrays			
•17.0692248	NM_010217.1	connective tissue growth factor	Ctgfl
•5.759602371	NM_012519.1	calcium/calmodulin-dependent protein kinase II,	Camk2d
•3.253212985	NM_027015.1	ribosomal protein S27	Rps27
•4.516116188	NM_010884.1	N-myc downstream regulated 1	Ndr1
•9.522688101	NM_009263.1	secreted phosphoprotein 1	Spp1
•0.639719	NM_016980.1	ribosomal protein L5	Rpl5
•0.567912	NM_007631.1	cyclin D1	Ccnd1
•0.633432	NM_022891.1	ribosomal protein L23 \	Rpl23
•0.725151	NM_025592.2	ribosomal protein L35	Rpl35
••Clontech Arrays			
•4.232	NM_008843	prolactin induced protein	Pip
•1.651	NM_010638	Kruppel-like factor 9, basic transcription element binding protein 1	Klf9(Bteb1)
•2.399	NM_011361	serum/glucocorticoid regulated kinase	Sgk
•1.96225	NM_009681	adaptor-related protein complex AP-3, sigma 1	Ap3
•2.85725	NM_011461	Spi-C transcription factor (Spi-1/PU.1 related)	Spic
•0.629	NM_008557	FXYD domain-containing ion transport regulator 3	Fxyd3
•0.59975	NM_009335	transcription factor AP-2, gamma	Ap2

•

Figure 10. Northern Blot of HC11 RNA following induction of lactogenic differentiation. RNA was isolated from HC11 cells induced to diffentiate with lactogenic hormone at various time post hormone addition. The blots were hybridized with cDNA probes from genes that were up-regulated as determined by array analysis. Quantitation of the hybridization results was performed and normalized to the actin signal.

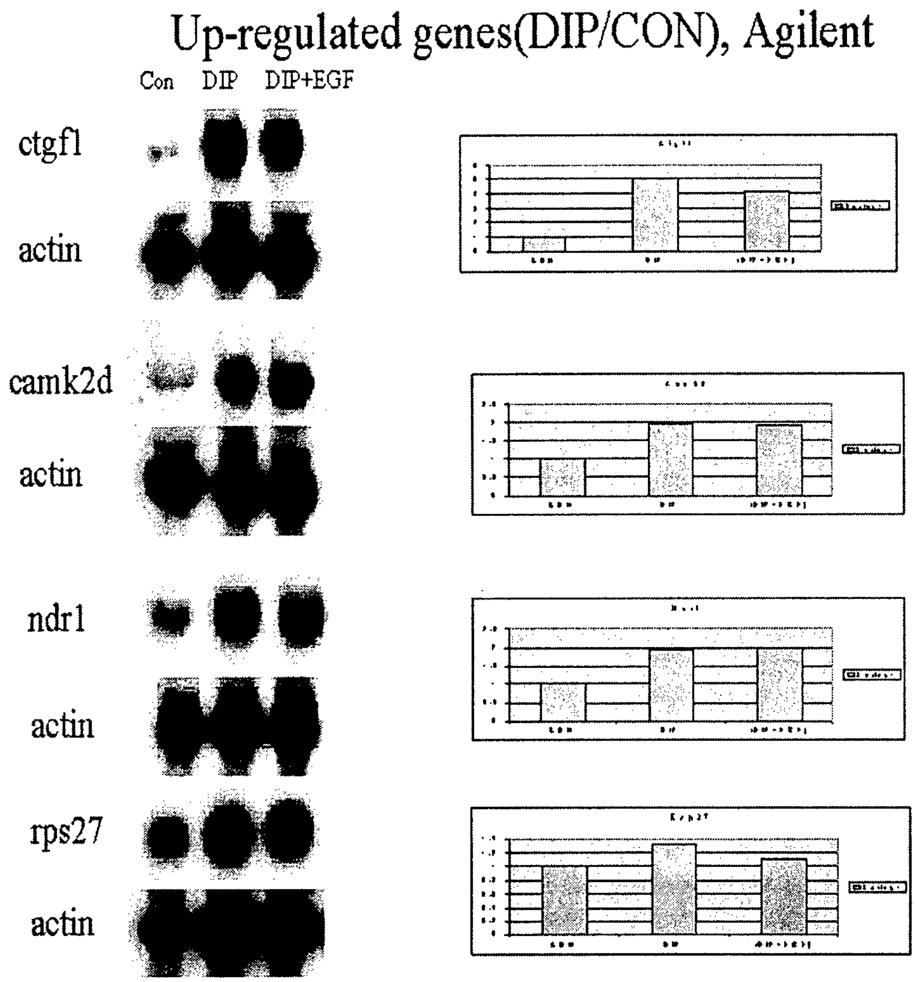

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Table 10 (Continued)

Up-regulated genes (Clontech, DIP/Con)